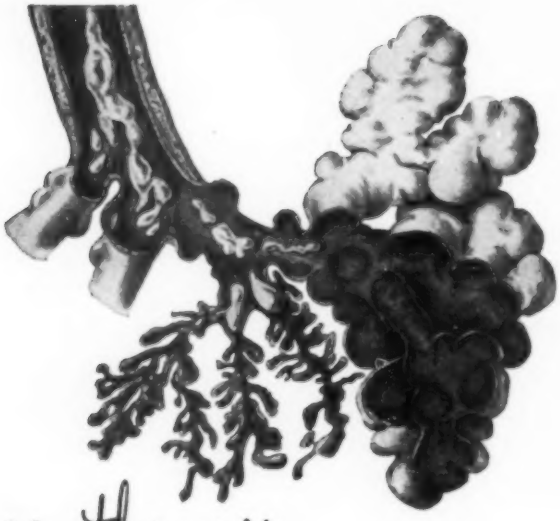
DECEMBER 1961
VOLUME 84
NUMBER 6

THE AMERICAN REVIEW OF RESPIRATORY DISEASES

OFFICIAL JOURNAL OF THE AMERICAN THORACIC SOCIETY
Medical Section of the National Tuberculosis Association



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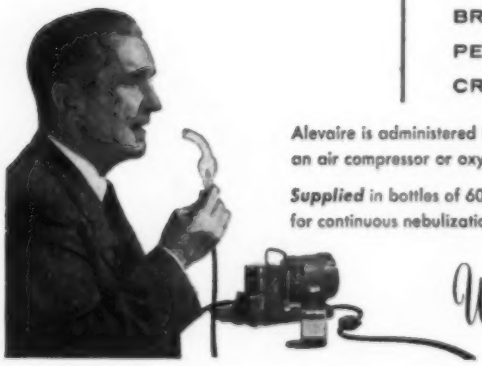

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THE AMERICAN REVIEW OF RESPIRATORY DISEASES

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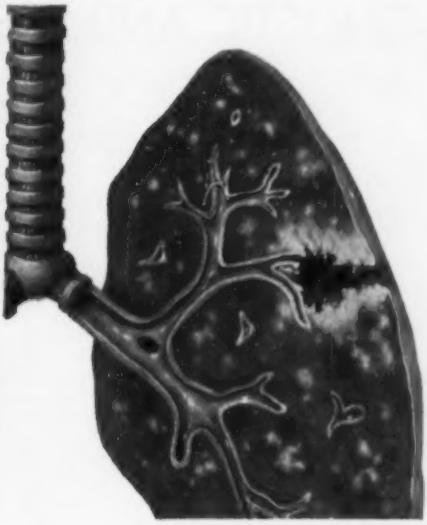

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1. Davey, W. N.: GP 19:107, No. 1, 1959.

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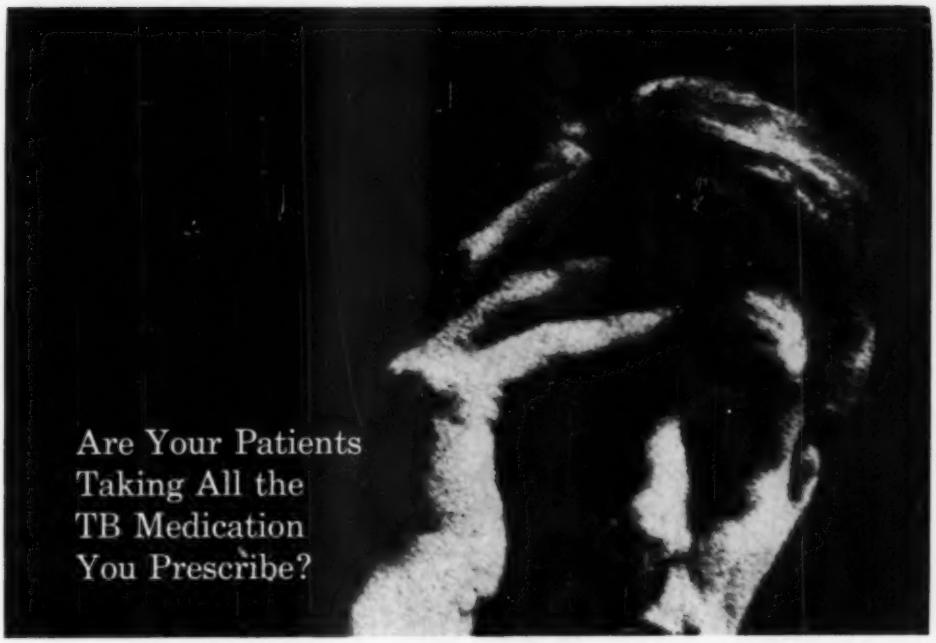

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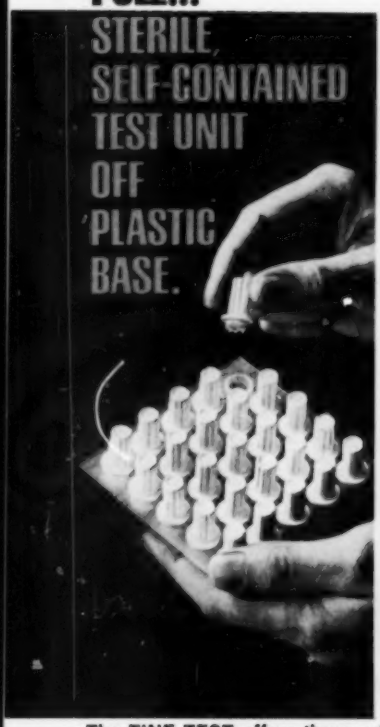

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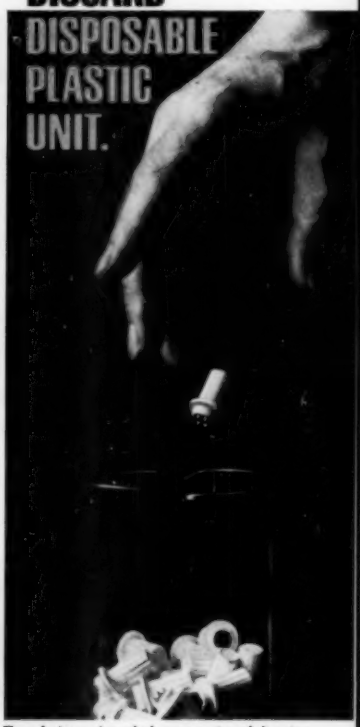

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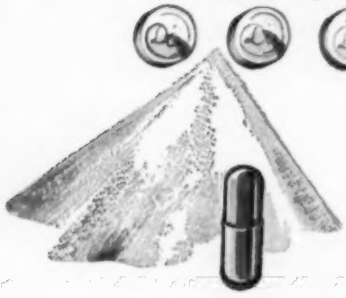

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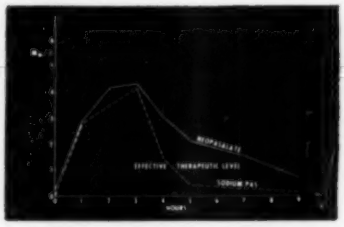

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ERRATUM

In the July, 1961, issue of the REVIEW (vol. 84), in the article entitled "The Effect of BCG Vaccination on the Fate of Virulent Tubercle Bacilli in Mice" by François M. Lévy *et al.*, there was a transposition of figures 2 and 3.

Figure 2 illustrates the *spleen* of the *control mouse*.

Figure 3 illustrates the *lung* of the *vaccinated mouse*.

THE AMERICAN REVIEW
OF RESPIRATORY DISEASES

Clinical and Laboratory Studies of Tuberculosis and Respiratory Diseases

VOLUME 84 *December 1961* NUMBER 6

CLINICAL USEFULNESS OF THE SINGLE-BREATH PULMONARY DIFFUSING CAPACITY TEST[1, 2]

BENJAMIN BURROWS, JOHN E. KASIK, ALBERT H. NIDEN, AND WILLIAM R. BARCLAY

(Received for publication April 6, 1961)

Introduction

During the past decade, there has been considerable interest in measurement of the pulmonary diffusing capacity (D_L), leading to the development of a variety of methods for its determination. These techniques have proved highly valuable in investigating the normal and abnormal physiology of the lung. Numerous reports have indicated that the diffusing capacity may be decreased by a variety of disease states, but the specificity of a low pulmonary diffusing capacity (D_L) for any particular type of lung disease has not been determined, nor have the normal limits for this capacity been definitely established.

The present report is a review of the writers' experience with one of the methods for determining pulmonary diffusing capacity, the Forster modification of the Krogh single-breath test (1). Results in 555 subjects have been tabulated in an attempt to determine the normal limits of the test, its ability to distinguish normal from abnormal subjects, its specificity for certain lung disorders, and its contribution to the total evaluation of lung function. In addition, the reproducibility of the test is evaluated.

Materials and Methods

Since 1957, single-breath diffusing capacity determinations have been performed as a clinical service in the Pulmonary Function Laboratory of the University of Chicago Clinics. Patients referred for a "complete evaluation" have received this test in addition to determination of their lung volume and nitrogen washout curve, simple tests of ventilatory mechanics, and ear oximetry. More than 2,000 patients were seen in the laboratory during this period of time, but only 555 subjects had D_L determinations, indicating some case selection. However, no conscious attempt was made to select cases for an eventual report on the diffusing capacity. For this reason, many of the patients whose cases are reported suffer from mild disease, and some have doubtful diagnoses. They are fairly representative of the type of patient referred for pulmonary function testing in a general hospital; many were tested as part of a preoperative evaluation. All subjects with diffusing capacities who were eighteen years of age or older have been included in the present report.

Residual volumes were determined in all subjects by a modification of the Darling open-circuit technique (2). The diffusing capacity was determined by the method of Forster and associates (1), using a Cambridge catherometer for helium analysis and a Liston Becker infrared absorption meter for carbon monoxide determination. For a period of six months, a Godart katapherometer was substituted for the Cambridge helium analyzer. The inspired mixture consisted of 0.25 to 0.30 per cent carbon monoxide and 9.0 to 14.0 per cent helium in air. Breath-holding times were kept between ten and twelve seconds. All reported values for D_L and for D_L per liter of lung volume (D_L/V_L) represent an average of at least two separate determinations. Values for lung volume are reported as standard temperature pressure dry (STPD), and values for the diffusing capacity are reported as cubic centimeters of carbon monoxide diffused per minute per millimeter of mercury alveolar-capillary gradient.

Ear oximetry was performed on almost all subjects in the study. For purposes of this paper, only the changes in arterial oxygen saturation with exercise were analyzed. Waters Corporation oximeter ear pieces were used throughout, in conjunction with a Grass oximeter control box, amplifier, and recorder. Both single-scale and double-scale readings were obtained, and the latter were used

[1] From the Department of Medicine, University of Chicago; and the Pulmonary Function Laboratory, University of Chicago Clinics, Chicago, Illinois.

[2] Supported by a research grant from the Illinois Tuberculosis Association.

for final determination of the change in arterial oxygen saturation. Exercise was performed by stepping on and off a nine-inch platform, a standard test consisting of thirty steps in a period of ninety seconds. Patients were allowed to stop at any time that dyspnea became severe or if there were a greater than 10 per cent fall in arterial oxygen saturation. For this reason, the quantity of fall noted is only a rough index of the blood gas disturbance, and exercise may not be strictly comparable from subject to subject. Falls in oxygen saturation of 2 per cent or less are considered insignificant in this laboratory and have not been analyzed.

Forty-one subjects had been referred for baseline tests prior to exposure to potentially toxic inhaled substances or because of psychogenic cardiorespiratory symptoms. An additional 25 patients were found to be free of all pulmonary disease and had completely normal pulmonary function tests (normal residual volumes, nitrogen washout curves, ventilatory mechanics, ear oximetry, and a vital capacity of at least 100 per cent of predicted by the Baldwin formula (3)). Sixty-nine patients had insignificant or strictly localized pulmonary lesions (such as "coin lesions") and had essentially normal pulmonary function tests (normal residual volumes, nitrogen washout curves, ventilatory mechanics, ear oximetry, and a vital capacity of at least 90 per cent of predicted). Data in the three groups were compared, and the results in these 135 subjects (84 men and 51 women, ranging in age from eighteen to eighty-nine years) were used to calculate the normal limits of pulmonary diffusing capacity for this laboratory.

The remaining subjects were then classified according to disease and the data analyzed. Diagnoses were based on review of the patients' charts at the time of testing or shortly thereafter, and diagnoses were not reviewed again for the purposes of this paper. An exception to this was the patients with congenital heart disease whose charts were restudied in order to delineate a group with high pulmonary blood flows proved by cardiac catheterization. It is possible that some diagnostic errors have been made, but in almost all instances the diagnoses were reached only after extensive clinical studies and after consultation with a specialist in the field concerned.

The "diffuse fibrosis" group consisted of 22 patients with undiagnosed or idiopathic pulmonary fibrosis, 12 patients with collagen diseases, and 2 patients with beryllosis. No other pneumoconioses and no infectious granulomas have been included. The post-resection group was composed of patients who have had all significant pulmonary disease surgically removed; the group was heavily weighted with post-pneumonectomy patients or other extensive pulmonary resections. The emphysema group consisted of patients with idiopathic chronic obstructive emphysema; "secondary" emphysema has not been included in this group.

Results

Control Group

Test results in the three groups not expected to have any abnormality in diffusion (41 normal subjects, 25 patients free of pulmonary disease, and 69 patients with localized pulmonary lesions and normal pulmonary function tests) were analyzed and revealed no differences between the groups. Data from the three groups were then combined as a single control population of 135 subjects, yielding the following relationships for pulmonary diffusing capacity (D_L) versus age, D_L versus body surface area (BSA), and D_L versus lung volume (V_L).

Relationship of D_L to age and sex: The correlations of total D_L with age for males and females, respectively, are revealed in figure 1*A* and *B*. Regression lines for these data, determined by the method of least squares, reveals

$$D_L = 36.7 - 0.238 \times \text{age} \quad \text{for men, and}$$

$$D_L = 25.0 - 0.117 \times \text{age} \quad \text{for women,}$$

when age is expressed in years. The standard error of the estimate of D_L from these equations is ± 5.08 for men and ± 4.53 for women. It is apparent that there is a significantly higher total D_L for men than for women at any given age. The effect of age on D_L is more marked in men than in women ($P < 0.01$ of chance occurrence as calculated by Student's t test), but the correlation of over-all D_L with age is significant in both men and women; the probability that the apparent effect of age on D_L is due solely to chance is less than 0.01 for both sexes.

Relationship of D_L to body surface area (BSA): The differences in total D_L in men and women of the same age can be ascribed largely to differences in body size, as noted in figure 1*C*. The regression line for D_L versus body surface area as determined by the method of least squares for all age groups is $D_L = 15.5 \times \text{BSA} - 4.6$, when BSA is expressed as M^2. The standard error of this equation is large, ± 5.89, but the correlation of D_L with body surface area is highly significant ($P < 0.01$ that the apparent relationship is due solely to chance).

Relationship of D_L to BSA, age, and sex: Combining the above regression equations yields:

$$D_L = 15.5 \times BSA - 0.238 \times age + 6.8$$ for men, and

$$D_L = 15.5 \times BSA - 0.117 \times age + 0.5$$ for women.

The standard error of the estimate of D_L from these equations remains large, ± 5.04. The relationship of D_L as measured to D_L as predicted from the above equations is depicted in figure 1*D*. The limits of two standard errors are also shown in this figure, and it will be noted that no normal value falls below the two standard error range. For the purposes of this paper, the ± 2 standard error range will be accepted as the limit of normal. Examination of figure 1*D* also reveals that the spread of D_L values is almost as great in the low diffusing capacity range as in the higher ranges. For this reason, it is unwise to express the limits of normal as per cent of predicted D_L; the absolute limit of ± 2 standard error (± 10.08) gives a narrower estimate of normal range.

Relationship of D_L to lung volume (V_L): There is a definite correlation between D_L and V_L ($P < 0.01$). The regression equation derived for the entire control group by the method of least squares is $D_L = 3.46 \times V_L + 7.0$ (± 3.4), when V_L is expressed in liters standard temperature pressure dry and is the lung volume during the breath-holding D_L determination.

The least-squares regression equation for diffusing capacity versus lung volume indicates a positive value for D_L when V_L equals zero. However, since the assumption of a uniform D_L/V_L ratio for a given age greatly simplifies calculations and is a useful concept, this approximation has been accepted and utilized in the remainder of the paper. The error involved in this approximation for the thirty- to forty-nine-year-old age group is indicated in figure 1*E*. It should be noted that there is no significant difference between the least-squares regression line (broken line) and the solid line which indicates the uniform D_L/V_L ratio in this age group ($P = 0.11$ that the difference between these lines is due solely to chance). Thus, the assumption of a uniform D_L/V_L ratio for a given age is not totally unreasonable.

Relationship of D_L/V_L to age: If the concept is accepted of a D_L/V_L ratio characteristic of a given age regardless of the absolute value of V_L, it is possible to plot D_L/V_L versus age (figure 1*F*). The least-squares regression line indicates that $D_L/V_L = 6.49 - 0.0298 \times age$ (± 1.12), and the correlation of D_L/V_L with age is highly significant ($P = <0.01$). It is apparent from figure 1*F* that there is no significant difference between D_L/V_L for men and for women of the same age. In other words, the difference in total pulmonary diffusing capacity between the sexes may be explained by their different lung volumes. The limits of two standard deviations noted in figure 1*F* will be accepted as the normal range for D_L/V_L in the laboratory. Values above and below two standard deviations will be considered high and low, respectively, while values between $+1$ and $+2$ standard deviation will be considered high normal and values between -1 and -2 standard deviation will be considered low normal. Values between $+1$ and -1 standard deviation will be called normal in the remainder of the paper.

Total D_L in Abnormal Subjects

The deviation from predicted of the total pulmonary diffusing capacity for all subjects studied is set forth in figure 2. It will be noted that those diseases which produced a loss of lung volume were associated with some fall in D_L, while the high pulmonary blood flow of congenital heart disease with left-to-right shunt produced D_L's which were higher than predicted. Patients with clinical diagnoses of bronchitis or asthma showed no significant change in over-all D_L, regardless of their degree of expiratory slowing at the time of testing. Acquired heart disease, bronchogenic carcinoma, and miscellaneous pulmonary disorders with vital capacities greater than 75 per cent failed to show a significant abnormality in D_L, while these same disorders were associated with a significant fall in diffusing capacity when lung volume was impaired. The diagnosis of chronic obstructive emphysema was associated with a significant fall in D_L, but the alteration was not as great as in those diseases which cause a reduction in lung volume. The dependence of total diffusing capacity upon lung volume became more apparent in studies of the individual groups. In figure 3, D_L was plotted against V_L for those patients with sarcoidosis, and a definite correlation of D_L and V_L was apparent. This correlation was less marked in patients with emphysema and in patients with diffuse fibrosis but was noted in all groups, even when the loss of

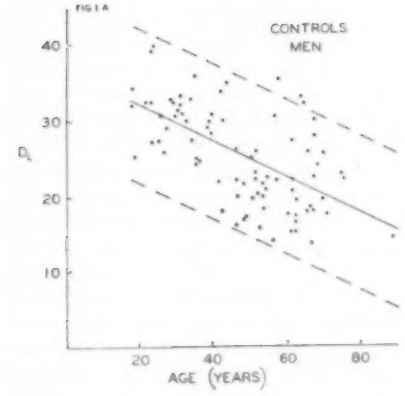
FIG 1A
CONTROLS
MEN
D_L
40
30
20
10
20
40
60
80
AGE (YEARS)

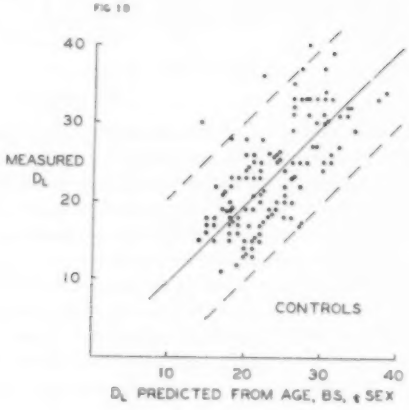
FIG 1D
MEASURED D_L
40
30
20
10
CONTROLS
10
20
30
40
D_L PREDICTED FROM AGE, BS, & SEX

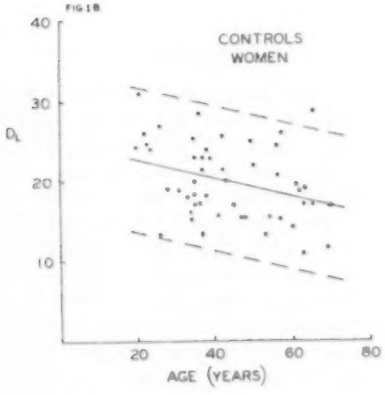
FIG 1B
CONTROLS
WOMEN
D_L
40
30
20
10
20
40
60
80
AGE (YEARS)

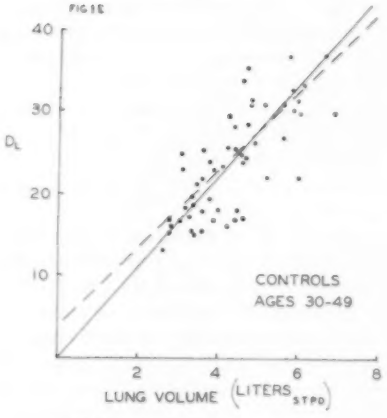
FIG 1E
D_L
40
30
20
10
CONTROLS
AGES 30-49
2
4
6
8
LUNG VOLUME (LITERS STPD)

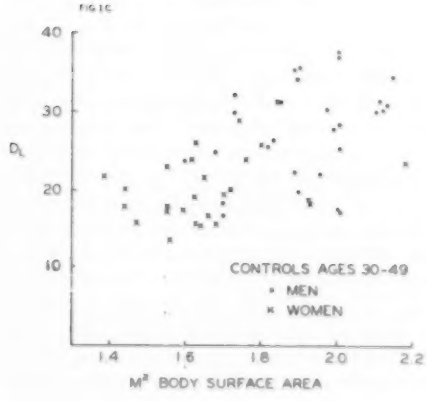
FIG 1C
D_L
40
30
20
10
CONTROLS AGES 30-49
• MEN
× WOMEN
1.4
1.6
1.8
2.0
2.2
M^2 BODY SURFACE AREA

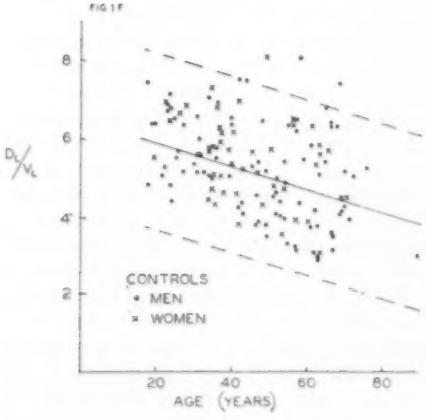
FIG 1F
D_L/V_L
8
6
4
2
CONTROLS
• MEN
× WOMEN
20
40
60
80
AGE (YEARS)

lung volume was due to simple surgical excision of pulmonary parenchyma.

It is apparent from figure 2 that a reduced diffusing capacity was a completely nonspecific finding, but the differences in V_L in the various diseases depicted made interpretation difficult. In figure 4, only patients with vital capacities between 60 and 80 per cent of predicted are shown in order to make the various groups more comparable. It would appear that all disorders showed a roughly comparable fall in diffusing capacity, with the exception of the asthmatic and bronchitic group.

The wide range of normal for pulmonary diffusing capacity results in a poor differentiation of normal from abnormal with this test. Even in patients with impaired vital capacities, a minority showed a definitely low D_L (below -2 standard error from predicted). In the 60 to 80 per cent vital capacity group (excluding subjects with asthma and bronchitis), only 23 per cent of the values for D_L were definitely low, 28 per cent were in the low normal range, 3 per cent were high normal, 1 per cent were above normal, and 45 per cent were entirely normal. With vital capacities above 80 per cent, a definitely low D_L was encountered in only 9 subjects in the entire series (3 with diffuse fibrosis, 2 each with metastatic neoplasms and with emphysema, and one each with sarcoidosis and with asthma), an incidence of less than 3 per cent; there were no clinical features which distinguished these 9 patients from others with the same diagnoses. In patients with vital capacities of less than 60 per cent of predicted, a low D_L was encountered 40 per cent of the time. Of patients with a definitely low D_L, 48 per cent had vital capacities of less than 60 per cent predicted; 38 per cent, vital capacities of 60 to 80 per cent of predicted; and only 14 per cent had vital capacities greater than 80 per cent, despite the fact that 67 per cent of all patients belonged in this last group. Thus, there was a high order of correlation between vital capactiy and D_L.

D_L/V_L in Abnormal Subjects

Deviations from predicted in the diffusing capacity per liter of lung volume are depicted in figure 5. The increase in diffusion in patients with high pulmonary blood flow is even more apparent when expressed per liter of lung volume than when expressed as total pulmonary diffusing capacity. Patients with acquired heart disease, asthma, bronchitis, or miscellaneous disorders did not show significant alteration in D_L/V_L. When analyzed by age, it is found that post-resection patients of less than fifty years showed an average increase in D_L of 0.76, while age groups fifty to sixty-four and sixty-five plus showed changes of $+0.29$ and -0.35, respectively. The increase in D_L/V_L after resectional surgery is significant in patients less than fifty years of age ($P < 0.05$).

Pulmonary parenchymal lesions produced a somewhat reduced D_L/V_L, with comparable alterations produced by sarcoidosis, diffuse fibrosis, and bronchogenic carcinoma. The most marked reduction in D_L/V_L was noted in emphysema, and this value would seem to be somewhat more reliable in distinguishing emphysema from bronchial asthma than was the over-all D_L, since 33 per cent of emphysematous patients had definitely low D_L/V_L, and an additional 36 per cent were in the low normal range. Only 5 per cent of bronchitic and asthmatic patients

FIG. 1. Data for the control group.

A and *B:* D_L versus age for men and women, respectively. The solid lines represent regression equation fitted by the method of least squares. Broken lines indicate the limits of two standard deviations.

C: D_L versus M^2 body surface area for age group thirty to forty-nine, indicating similar D_L values for men and women when body surface area is controlled.

D: Measured versus predicted D_L. The solid line represents the line of perfect agreement between measured and predicted values. Broken lines indicate the limits of two standard deviations of the data.

E: D_L versus liters of lung volume for the thirty- to forty-nine-year age group. The least-squares regression line for this data is indicated by the broken line. The solid line has been visually fitted, and would indicate a uniform D_L/V_L ratio for this age group. (*See* text for further explanation.)

F: D_L/V_L versus age; solid line represents the regression line determined by the method of least squares, and the broken line, the limits of two standard deviations.

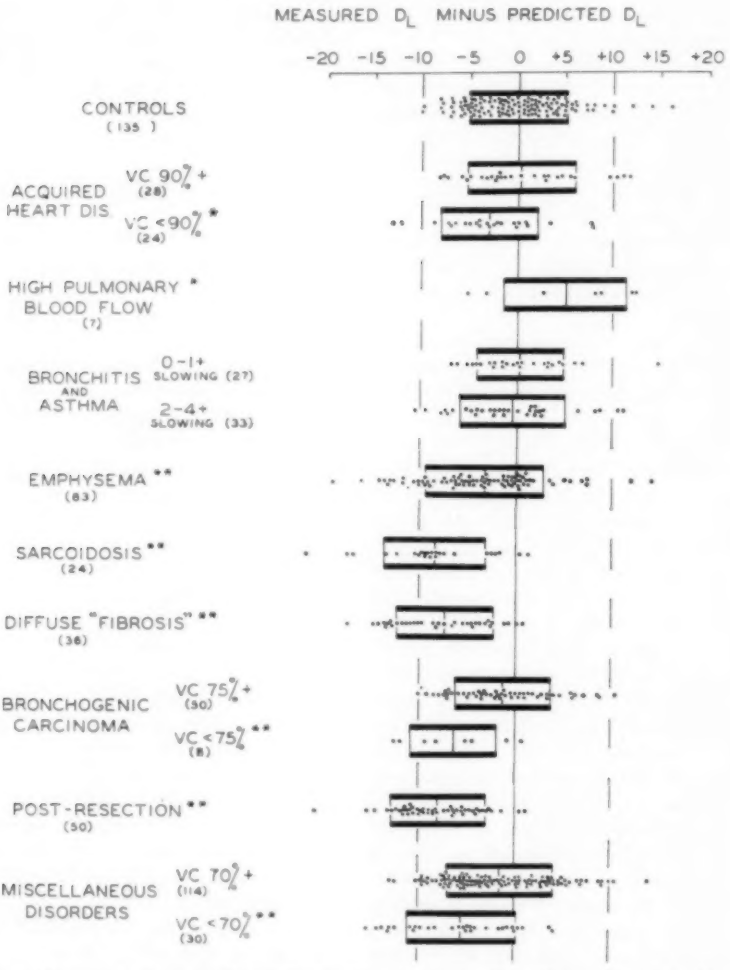

FIG. 2. Measured minus predicted D_L for all patients studied. The number of subjects in each diagnostic group is indicated in parentheses following the name of each group. Each subject is represented by a single dot, and the average value for each group is indicated by a vertical line. Horizontal bars above and below this average line indicate the limit of one standard deviation for each group. The vertical broken lines indicate the limits of two standard deviations from the control group, and therefore, the limits of normal range. *Values significantly different from the control group at $P < 0.05$. **Values significantly different from the control group at $P < 0.01$.

had definitely low D_L/V_L, and 15 per cent were low normal.

Other than distinguishing emphysema from bronchitis, the D_L/V_L would seem to have little differential diagnostic value. Considering only subjects with vital capacities of 60 to 80 per cent of predicted (figure 6), it is apparent that the spread of data is too great to be of significance in an individual case, despite the lower average D_L/V_L values in diffuse fibrosis and in emphysema than in the other pulmonary disorders. Also, determination of D_L/V_L would not appear an adequate test to separate normal from abnormal subjects since only 33 per cent of the

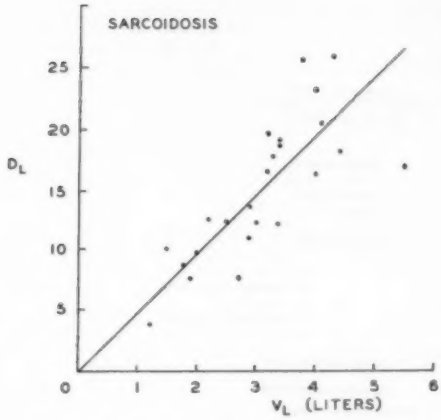

FIG. 3. D_L versus V_L in patients with sarcoidosis. The solid line indicates a visually fitted regression line corresponding to a uniform D_L/V_L ratio for patients with this disease.

emphysematous patients and only 4 per cent of all other abnormal subjects were definitely abnormal by this parameter.

Correlation of D_L with Arterial Oxygen Desaturation on Exercise

In analyzing the blood gas changes associated with a reduced D_L, only the fall in arterial oxygen saturation on exertion has been considered. It is apparent that there was a definite correlation of D_L with hypoxemia on exertion; 75 per cent of subjects with a D_L of less than 7.5 showed a significant fall in arterial oxygen saturation, and in 63 per cent there was a fall of 5 per cent or more. Only 9 per cent of patients with diffusing capacities in excess of 22 showed a comparable fall in arterial oxygen saturation.

Reproducibility of the Single-Breath Diffusing Capacity Test

The average discrepancy between successive breath-holding diffusing capacity tests was 1.03 (average deviation from the mean of 0.51 and a coefficient of variation between tests of 6 per cent). In 6.4 per cent of cases, the difference between successive tests was greater than 2.5, and in only 1.7 per cent did such values disagree by more than 5.0. The test was equally reproducible in patients as in the control group, even in the presence of emphysema.

DISCUSSION

There has been general agreement that the diffusing capacity is dependent upon body size (4–9). Usually, body surface area has been used as an index of body size because of its known relationship to oxygen consumption; it has been tacitly assumed that D_L should be related to the latter parameter. However, D_L has also been shown to be related to height and weight (6, 9). The equation relating D_L and BSA ($D_L = 15.5 \times BSA - 4.6$) is similar to that of Marks and associates ($D_L/BSA = 16.5$) (10), is in reasonable agreement with that of Ogilvie and associates (5) ($D_L = 18.85 \times BSA - 6.8$), but is somewhat

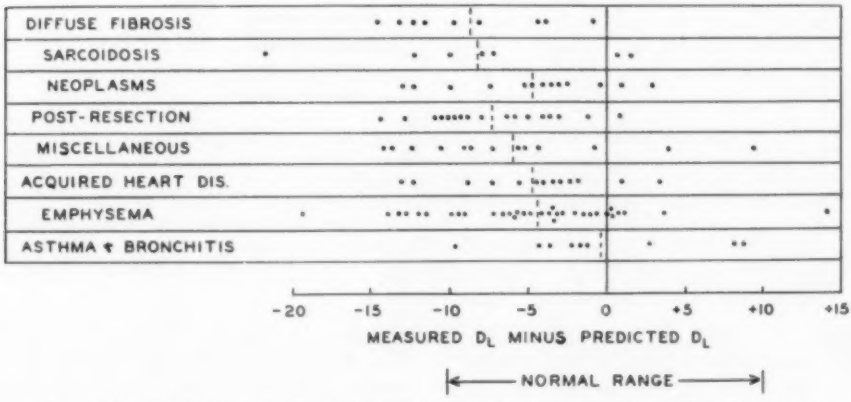

FIG. 4. Measured minus predicted D_L in patients with vital capacities of 60 to 80 per cent. Vertical broken lines indicate average values for each group.

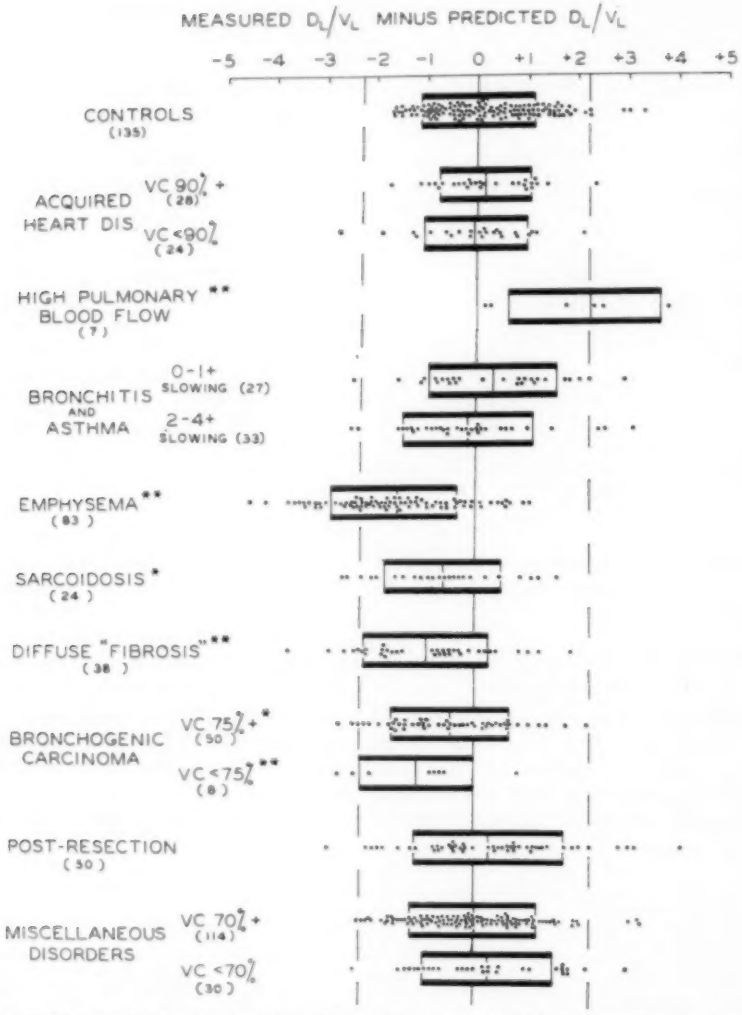

FIG. 5. Measured minus predicted D_L/V_L for all patients studied. The number of subjects in each diagnostic group is indicated in parentheses following the name of each group. Each subject is represented by a single dot, and the average value for each group is indicated by a vertical line. Horizontal bars above and below this average line indicate the limit of one standard deviation for each group. The vertical broken line indicates the limits of two standard deviations for the control group, and therefore, the limits of normal range. *Values significantly different from the control group at $P < 0.05$. **Values significantly different from the control group at $P < 0.01$.

different from that of McGrath and Thomson (7) who found that $D_L = 24.25 \times BSA - 0.289 \times age - 3.4$. At the age of forty and with 1.8 M^2 BSA, the writers' equation, Ogilvie's, and McGrath and Thomson's yield values of 23.3, 27.1, and 28.6, respectively. The lower predicted D_L from the present formula may be due to a difference in selection of the control group. Control subjects in the present series consisted of patients referred for pulmonary function tests who were

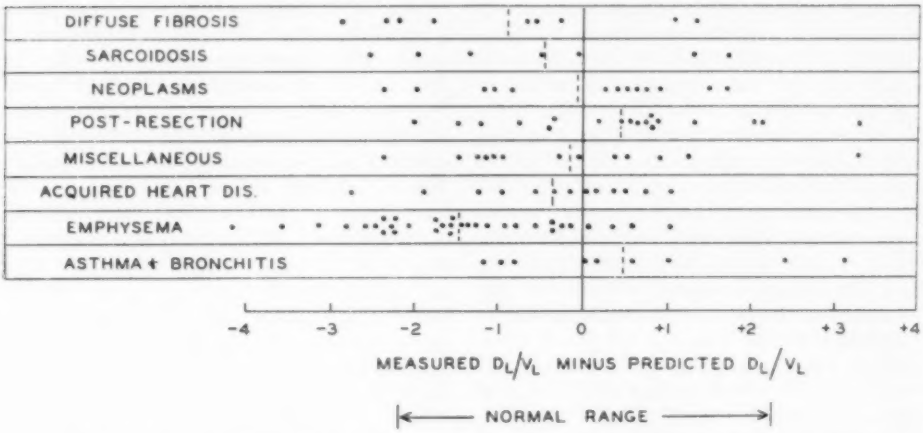

FIG. 6. Measured minus predicted D_L/V_L in patients with vital capacities of 60 to 80 per cent. Vertical broken lines indicate average values for each group studied.

found to have normal cardiopulmonary systems, while, in other series, strictly normal persons were selected. It is possible that subjects selected as strictly normal represent the best physiologic status for their size and age rather than an average value applicable to a patient population. In selected normal subjects used for physiologic research in this laboratory, the D_L's have been systematically slightly higher than those predicted by the present regression formula. However, it would seem more reasonable to use values obtained in patients with normal lungs as a basis for comparison with patients with pulmonary disease than to compare the latter with an ideal physiologic group.

It is clear from the present data that pulmonary diffusing capacity is age dependent. Although some earlier studies found no significant variation in D_L with age, leading to the conclusion that resting D_L is not age determined (4, 5, 8, 11, 12), the age range studied was small and the effects of body size were not taken into account. Some other recent studies have revealed a definite fall in diffusing capacity with advancing years. A fall in breath-holding D_L of 0.238 per year of age is noted in men in this series. Using the same test, McGrath and Thomson noted a fall in D_L of 0.289 per year of age (7). With steady-state techniques, a significant decrease in D_L with age has also been reported (13, 14). That the effect of age is independent of body size in this series becomes apparent when least-squares regression lines for various age groups are calculated for D_L versus body surface area. A systematic decrease in D_L with age may be noted at any given body size. This effect of age is very likely related to degenerative changes in the lungs and pulmonary vasculature, changes which would progress at quite variable rates in different persons. This probably accounts for the wider range of normal D_L values in older than in younger subjects in this series (*see* figures 1.1 and *F*). Failure to recognize the effects of age in some early studies could have been due, in part, to case selection. "Physiologically young" persons would very likely be selected to represent the older age group in a study of strictly normal subjects; such persons might show only minimal effects of aging.

The prediction formula for single-breath diffusing capacity in men based on age and body size for the present data is $D_L = 15.5 \times BSA - 0.238 \times age + 6.8$ and is somewhat at variance with the formula derived by McGrath and Thomson (7), $D_L = 24.246 \times BSA - 0.289 \times age - 3.4$. At the age of fifty-eight and with 1.5 M^2 body surface area, these formulas predict identical D_L's; but, with larger body size or at a younger age, McGrath and Thomson's formula predicts higher diffusion values. This could be due, in part, to case selection as discussed above.

The difference in effect of age on men and on women in this series is of interest. Similar data for women have not been found in the previous

literature with which to compare the present results. While men showed a decline in D_L of 0.238 per year of age, women showed a decline of only 0.117, and this difference was statistically significant. Whether or not this indicates some sex difference in the manner of aging of the lung and whether it may be related to the greater tendency toward emphysema in the male (and is not a peculiarity of case selection) is a matter for speculation. However, since the effect of age on D_L/V_L was not significantly different in men and in women, the apparent difference in age effect on total D_L may have no real meaning. On the basis of present data, it would appear necessary to apply a somewhat different prediction formula for D_L in women than in men, that is, $D_L = 15.5 \times BSA - 0.117 \times age + 0.5$.

Despite accounting for both body size and age, control subjects in this series showed a very wide scatter of pulmonary diffusing capacity around predicted. The high standard errors of the prediction formulas (± 5.0) indicate that as many as 5 per cent of normal subjects may have D_L's which vary from predicted by more than 10 cc. of carbon monoxide per minute per mm. of mercury. For small older subjects, normal D_L's range from approximately 10 to 30, while in the younger age groups and larger subjects, the range is about 20 to 40. Any test with such a wide physiologic range is apt to be difficult to apply in clinical medicine. The reasons for this extreme variability are probably multiple. The accuracy of the breath-holding test in measuring true over-all pulmonary diffusion has been questioned. Although available evidence suggests that it is reasonably precise in normal subjects (4, 15, 16), any inaccuracy in measurement might increase the variability of the test beyond its true physiologic limits. Also, breath-holding D_L has been found to be dependent upon metabolic state (4, 17), alveolar oxygen tension (18), alveolar carbon dioxide tension (19), and upon the degree of inflation of the lung at the time of breath holding (5, 10, 20, 21). These factors cannot be completely controlled in clinical application of the test, contributing to its variability from subject to subject. Finally, any technical errors would add to the spread of data, but this factor is probably relatively unimportant in view of the reproducibility of the procedure.

There is a definite correlation of D_L with lung volume (V_L) in the present series, and the concept of a characteristic D_L/V_L value for a given age regardless of sex or body size is suggested. The value D_L/V_L is especially useful in that it may be determined during a single-breath D_L determination without knowledge of actual V_L, eliminating the need for (and the errors of) a separate measurement of residual volume.[3] Diffusing capacity per liter of lung volume is related directly to Krogh's "permeability" k (7). Since $k = \ln (F_{ACO_0}/F_{ACO_t})/t$, it is apparent that $D_L/V_L = 1.4\ k$.[3] Normal values for k have been reported by McGrath and Thomson (7) who noted slightly less variability in k than in over-all D_L in their subjects. In the present series the coefficient of variability for D_L is 22 per cent and for D_L/V_L is 21 per cent, compared with values of 18 and 15 per cent, respectively, in McGrath and Thomson's data. These differences would not seem of practical importance. However, the value D_L/V_L might assume a particular usefulness when applied to abnormal lungs, since a fall in D_L that accompanied a corresponding fall in V_L, i.e., no change in the D_L/V_L ratio, could have a different significance than an equivalent fall in D_L not associated with as great a V_L alteration. In the latter instance there would be a decrease in D_L/V_L, perhaps indicative of loss of membrane area, reduced vascularization, or decreased membrane permeability in the remaining lung. Certainly, a true "alveolar-capillary block" should be accompanied by a fall in D_L/V_L in addition to a decrease in over-all diffusing capacity.

Normal values for D_L/V_L are noted to be definitely age dependent in this series ($D_L/V_L = 6.49 - 0.0298 \times age$), although the effect of age

[3] D_L may be calculated from the following equation when Pb is the millimeter of mercury barometric pressure, t is the time of breath holding in minutes, F_{ACO_0} is the alveolar concentration of carbon monoxide at the onset of breath holding, and F_{ACO_t} is the alveolar carbon monoxide concentration at the end of time t:

$$D_L = \frac{V_{L(co)}}{(Pb - 47)t} \times \ln \frac{F_{ACO_0}}{F_{ACO_t}}.$$

If V_L is expressed in liters, and with a Pb of 760 mm. of mercury, the equation may be written:

$$D_L/V_L = 1.4 \ln (F_{ACO_0}/F_{ACO_t})/t.$$

Thus, D_L/V_L may be determined directly from the fractional fall in alveolar carbon monoxide per unit time.

in the present subjects is somewhat less marked than in those reported by McGrath and Thomson (7). Converting the regression equation of those writers to D_L/V_L versus age, instead of k versus age, yields: $D_L/V_L = 8.1 - 0.053 \times$ age. At seventy years of age, the above formulas predict the identical D_L/V_L's; but, at younger ages, D_L/V_L is predicted higher by McGrath and Thomson's equation than by the one derived in this paper. This could be a result of differences in case selection. The dependence of D_L/V_L upon age might be regarded as further evidence of deterioration in the diffusion function of the lung with advancing years.

It is worth considering how this decrease in D_L/V_L with age might affect other methods for determining D_L. Since steady-state and rebreathing carbon monoxide techniques are performed with the lung near its functional residual capacity and since this volume is known to increase with age, it is possible that the larger lungs of older persons might yield a total D_L value similar to that of the smaller lungs of younger subjects, despite the higher D_L/V_L values in the latter group. Thus, the poorer diffusing characteristics of older lungs might be masked, at least in part, with either steady-state or rebreathing techniques.

Results in abnormal subjects in this series are in accord with previously published results in more selected patient material. No attempt will be made to review the voluminous literature pertaining to pulmonary diffusing capacity in disease. The reader is referred to a few recent review articles which summarize much of the reported data (4, 8, 11).

The high pulmonary blood flow associated with left-to-right congenital shunts produces a high pulmonary diffusing capacity (results similar to those of other investigators (*see* 35–37)) and is associated with a striking elevation in D_L/V_L.

The elevation in D_L/V_L in the present small series is sufficiently great to suggest that this parameter may be of some diagnostic value in congenital heart disease; certainly, finding a D_L/V_L more than 2.2 above predicted in a patient with a suspected congenital cardiac lesion would strongly suggest the possibility of a left-to-right shunt. The only other condition which produces a significant elevation in D_L/V_L in this series is the post-resection state in patients less than fifty years of age. Patients more than sixty-five years of age showed no increase in D_L/V_L following pulmonary resection. The apparent difference in the effect of pulmonary resection on younger and on older subjects may be related to a greater distensibility of the pulmonary vessels in younger subjects, allowing an increase in D_L/V_L when increased blood flow is induced in the remaining lung by excision of part of the pulmonary vascular bed. The elevated D_L/V_L noted with increased pulmonary blood flow is in accord with unpublished data from this laboratory in the perfused dog lung, but is not in agreement with some other observations.

Rosenberg and Forster (22) noted no independent effect of flow on D_L in the perfused cat lung. Ross and associates (23) and Turino and co-workers (24), on the basis of experiments in man, concluded that D_L is not blood-flow dependent. The true effect of blood flow on diffusing capacity would seem to be in doubt at the present time. It is possible that the D_L/V_L increase in young post-resection and congenital cardiac patients is not due to the increase in blood flow per se but to an associated alteration in pulmonary vascular tone or to an increase in pulmonary capillary transmural pressure; the latter is known to produce an increase in D_L (22, 25).

The slight increase in D_L/V_L after extensive pulmonary resection is not nearly sufficient to prevent a marked fall in over-all D_L with the loss of lung volume attending this procedure. A decrease in diffusing capacity after removal of portions of lung has previously been reported (26–28), and there appears to be a decrease in D_L/V_L in the early postoperative period with a later return toward normal (28). From results in patients presently reported (who are generally studied long after surgery), it would appear that D_L/V_L eventually rises above preoperative levels in younger subjects.

The fall in total D_L which accompanies the post-resection state is roughly equivalent to the fall noted in a variety of pulmonary disorders studied, especially in patients with comparable vital capacities. The spread of data is similar in all groups except emphysematous ones. It is not possible to separate a group of patients with definitely reduced diffusion from another group in whom D_L is well preserved in any of the diseases studied. On the contrary, the D_L values noted in pulmonary disorders associated with a low vital

capacity (VC) are compatible with a random reduction of diffusion in all subjects. Certainly, some normal D_L values are retained, but these could represent subjects whose normal D_L's were initially in the high normal range. This observation raises some question as to the specificity of the "alveolar-capillary block" syndrome and as to the ability of the single-breath diffusing capacity to delineate the syndrome. Present data suggest that loss of diffusion is a general development in pulmonary disease, and the correlation of D_L loss with vital capacity fall suggests that the degree of diffusion abnormality generally parallels the over-all extent of the disease. Of course, it is possible to compare subjects whose D_L is definitely low from sarcoidosis or diffuse fibrosis with other subjects who have similar diseases but normal pulmonary diffusing capacities; such comparisons might lead to the conclusion that there were two physiologic types of sarcoidosis or fibrosis whose average D_L's differed by 12 cc. of CO/min./mm. of mercury or more. On the other hand, a similar arbitrary separation of groups can be made in the control subjects, and two physiologic types of normal lungs can be described. Since data spread in a reasonably normal distribution, such a procedure is certainly unjustified in normal persons and is probably unjustified in pulmonary disease as well.

Much of the support for the concept of a distinctive syndrome of "alveolar-capillary block" has come from histologic studies revealing thickened alveolar septa or thickened alveolar linings. In the writers' experience, such changes, although striking, are usually of limited extent and alternate with areas of complete loss of alveolar structure, ischemia, microscopic atelectasis, and normal lung. Whether the physiologically important lesion in such lungs is actually a "block" between alveolus and capillary has not been demonstrated. Certainly, a comparable loss of diffusion may be encountered in subjects without such anatomic changes but with only a loss of alveolar-capillary membrane surface (as in the post-resection cases) or with only a pulmonary ischemia (as in anemia (29)). It seems reasonable, therefore, to consider the clinical syndrome of "alveolar-capillary block" as the end stage of any pulmonary destructive process in which airway obstruction or hypoventilation is not a prominent feature. The term "block" is very likely a misnomer in many of these patients whose difficulty would be more appropriately labeled "inadequate functioning pulmonary parenchyma." The diagnosis of this syndrome is not dependent solely upon the determination of D_L for, as noted by Forster (11), the diagnosis may be made with "reasonable surety" in the presence of a nonobstructive pulmonary insufficiency state accompanied by arterial unsaturation on exertion when such unsaturation is not due to shunting.

It is also important to note that there is not a clear difference between shunting and diffusion loss. If a "block" between alveolus and capillary becomes extreme (for example, with complete filling of the alveolus), blood flow to that region will reach the left heart unchanged by its passage through the lung and will produce a physiologic pattern resembling an anatomic shunt. Conversely, if a regional bronchial obstruction producing a physiologic shunt becomes complete, the area of lung supplied by that bronchus becomes useless as a diffusing structure, resulting in a lowering of D_L. In a "pure" defect, correction of arterial unsaturation should be regularly accomplished by the alveolar oxygen tension attained while breathing 30 per cent oxygen (30). In the experience of this laboratory, complete correction of significant arterial unsaturation on exertion on inhalation of 30 per cent oxygen is extremely rare, even in the presence of the characteristic clinical syndrome of "alveolar-capillary block." Thus, although diffusion loss may be the predominant physiologic abnormality in this syndrome, some degree of physiologic or anatomic shunting must generally exist.

There was a definite correlation between D_L and arterial hypoxemia, but this correlation was far from perfect. There were 47 patients in the present series without emphysema or anatomic shunts in whom a fall in arterial oxygen saturation of 5 per cent or more was noted on exertion. All of these patients qualified on clinical grounds and on pulmonary function tests as examples of the "alveolar-capillary block" syndrome. Since detailed studies of the oxygen gradient at different alveolar oxygen tensions were not carried out, it is impossible to determine the relative importance of physiologic shunting and of diffusion loss in producing the unsaturation in these subjects. However, a few of these patients had relatively normal diffusing capacities (as high as 24), while some showed diffusing capacities as low as 5 cc. of CO/min./mm. of mercury. This discrepancy could be due, in part, to the presence

of different amounts of associated physiologic shunting or relative hypoventilation, but the writers suspect that inaccuracies in the measurement of over-all D_L in these abnormal lungs are of major importance. It has been reported that gross under- or overestimation of D_L may occur with the breath-holding technique in abnormal lungs (16). The noted correlation of D_L and arterial unsaturation does not prove the reliability of the D_L measurement, as might be assumed. First of all, there are many gross individual discrepancies between D_L and hypoxemia. As important, arterial unsaturation is correlated with many indices of pulmonary dysfunction. For example, there is a definite correlation of fall in arterial oxygen saturation and vital capacity. Since D_L parallels a decrease in other parameters of lung function, some correlation of D_L with arterial oxygen saturation would be expected, regardless of the ability of D_L to measure the true diffusing characteristics of the abnormal lung.

The possible inaccuracies in measurement of D_L in abnormal lungs do not imply a lack of reproducibility of the test. If technical errors are minimized and if physiologic parameters can be duplicated, successive tests may show excellent agreement even if both values are grossly erroneous. Certainly, the breath-holding pulmonary diffusing capacity is highly reproducible on successive tests (coefficient of variation is 6 per cent in this series); the results in the present study are very similar to those of Ogilvie and associates in normal subjects (5), and there was no difference between the reproducibility of the test on the same day in normal and in abnormal subjects in this series. Although the data have not been completely analyzed, a greater variability has been noted in D_L values obtained weeks or months apart than of values obtained on the same day. In normal subjects, the reproducibility would still appear relatively good (as also noted by Ogilvie and associates); but in some abnormal subjects, especially with emphysema, widely divergent D_L values have been noted on tests performed weeks or months apart, even in the absence of other significant clinical or physiologic change. This could be due to subtle alterations in the function of the lung which change the direction or magnitude of the error in the D_L determination.

The most striking characteristic of the breath-holding pulmonary diffusing capacity in this study has proved to be its great physiologic variability. This seriously limits its usefulness in practical application. If used as a screening test for the presence of a pulmonary disorder, normal limits would have to be extended so widely as to delineate only a minority of abnormal subjects. Even with diffuse fibrosis producing a definitely reduced vital capacity, less than half the patients showed definitely reduced D_L. For such patients, in the writers' experience, the vital capacity proved a far better indicator of the presence of disease. Shepard and Turner (31) noted that the vital capacity was the best single test in delineating a group of subjects with an interstitial fibrosis and that additional pulmonary function tests did not add significantly to the ability of the vital capacity to distinguish normal from abnormal subjects. The present results are certainly in accord with their conclusions as far as the breath-holding D_L is concerned. Since there is no significant difference between D_L's in any of the pulmonary parenchymal diseases when the extent of the disease is taken into account, and since the overlap of data is enormous, the breath-holding D_L would seem to be of no differential diagnostic value in these disorders. However, as previously noted, it does seem to define a small group of congenital cardiac patients with an elevated pulmonary blood flow, especially if D_L/V_L is taken into account.

It has been reported that D_L is a useful measurement in differentiating bronchial asthma from obstructive emphysema (32) and that, even within the emphysematous group, patients with pre-existing bronchitis have higher diffusing capacities than those in whom bronchitis has not been a problem (33). Although there is a clear difference between groups of patients with emphysema and with asthma in this series, the spread of data is so great as to limit the diagnostic value of the D_L test in an individual patient. A distinctly low D_L would be strongly suggestive of frank emphysema, but only 14 per cent of emphysematous patients in this series showed so marked a loss of diffusion. Even a small selected series of well studied patients with "pure" emphysema, i.e., without significant associated bronchitis or bronchospasm, revealed only 8 per cent definitely low D_L's, although the average D_L was markedly reduced and 38 per cent of the tests were in the low normal range. In contrast to the findings of Williams and Zohman (34), there is a definite correlation be-

tween D_L and ventilatory capacity in emphysema in the writers' experience, despite a considerable spread of data. In a group of 60 well studied emphysematous patients, there were 29 with maximal breathing capacities of less than 30 liters per minute. These patients had an average D_L of 16.7, while patients with higher maximal breathing capacities had an average D_L of 22.6. A similar correlation of D_L with clinical and fluoroscopic assessment of the degree of emphysema was noted. However, no difference in D_L could be noted between subjects with or without bronchitis when patients with comparable degrees of emphysema were considered. It would be surprising if there were no correlation whatever between ventilatory disturbance and D_L impairment in emphysema, since it might be expected that both parameters would become more impaired as the disease progressed. This does not imply that the two functions may not be somewhat disassociated in certain persons. However, in view of the possible gross errors in measurement of D_L in emphysema (4, 15, 16), caution must be used in the interpretation of such data.

Interpretation of reports on diffusing capacity in disease is seriously hampered by the variety of methods for its determination. Since these techniques are all subject to error in an abnormal lung and since the direction and magnitude of these errors may vary from method to method in different diseases (4, 15), it is unreasonable to expect results with steady-state or rebreathing carbon monoxide techniques to correspond perfectly with those of the breath-holding method. This may account for some of the discrepancy between the present results and reports of some other investigators. It is possible that steady-state techniques which measure D_L on exertion have a higher specificity for disease than the single-breath technique at rest. However, on the basis of the writers' experience with the breath-holding method, it must be concluded that this test is of little practical usefulness in a clinical pulmonary laboratory. Its variability is too great to justify its use as a screening procedure for the presence of pulmonary disease in an individual patient, and a decrease in D_L is too general an accompaniment of pulmonary disease to make it useful in differential diagnosis. In the writers' experience, decrease in D_L generally parallels falls in other indices of pulmonary function, and individual discrepancies may be explained by the wide variability of data. However, a different conclusion may be reached if small, selected populations are studied, and especially if the effects of age, body size, and lung volume are not taken into account. In order to demonstrate a unique value of the pulmonary diffusing capacity test, it must be shown that there is a significant difference between the disease of patients with differing diffusing capacities but with the same diagnosis, same lung volume loss, and same ventilatory impairment. Such demonstration requires detailed double-blind anatomic-physiologic correlations; as yet, such studies are not available.

At the present time, it must be concluded that the expense and inconvenience entailed in using the breath-holding D_L test are not justified solely on the basis of the clinical usefulness of the procedure. This is not meant to detract from the usefulness of the test in teaching or from its demonstrated effectiveness as a research tool in both normal and abnormal pulmonary physiology, nor may one infer from this anything about the possible clinical usefulness of other methods for determining pulmonary diffusing capacity.

Summary

The breath-holding pulmonary diffusing capacity (D_L) was found to be dependent upon body size and age in a group of 135 patients without significant cardiopulmonary disease. Regression formulas for predicting pulmonary diffusing capacity have been determined:

$$\text{For men: } D_L = 15.5 \times \text{body surface area} - 0.238 \times \text{age} + 6.8;$$

$$\text{For women: } D_L = 15.5 \times \text{body surface area} - 0.117 \times \text{age} + 0.5.$$

The concept of a characteristic pulmonary diffusing capacity/lung volume (D_L/V_L) ratio for a given age is suggested, and a regression formula applicable to both sexes has been determined:

$$D_L/V_L = 6.49 - 0.0298 \times \text{age}.$$

Even when age, sex, and body size are taken into account, there is very marked variability in pulmonary diffusing capacity and pulmonary diffusing capacity/lung volume from subject to subject. This variability could not be accounted for by any parameter studied.

The diffusing capacity results in 555 patients are reported. The pulmonary diffusing capacity was decreased in all cardiopulmonary disorders studied when accompanied by a fall in lung

volume, and it was reduced in pulmonary emphysema. However, the pulmonary diffusing capacity was not appreciably altered in asthma or bronchitis without associated emphysema.

Because of wide variability of data, the usefulness of the breath-holding pulmonary diffusing capacity test is extremely limited in determining the presence of disease or in differential diagnosis. It may have some usefulness in distinguishing emphysema from asthma or bronchitis, especially if the pulmonary diffusing capacity/lung volume is considered, although a low breath-holding diffusing capacity certainly cannot be considered a necessary criterion for the diagnosis of emphysema.

A high pulmonary blood flow in a young person was found to be associated with some increase in pulmonary diffusing capacity/lung volume, and this test may have a limited usefulness in the diagnosis of left-to-right shunt in congenital heart disease.

The specificity of the "alveolar-capillary block" syndrome for any particular group of diseases is questioned, as is the aptness of the term "block" for many of the subjects in whom this syndrome is diagnosed. The term "inadequately functioning pulmonary parenchyma" would seem more generally applicable.

The excellent reproducibility of the breath-holding pulmonary diffusing capacity determination on successive tests is confirmed.

A definite correlation between the pulmonary diffusing capacity and arterial unsaturation on exertion was noted, but many individual discrepancies occurred. A significant fall in oxygen saturation on exertion generally occurred with diffusing capacities of less than 8 cc. of carbon monoxide per minute per mm. of mercury.

The expense and inconvenience of the single-breath diffusing capacity test do not seem justified solely on the basis of its clinical usefulness, and caution must be exercised in the interpretation of breath-holding pulmonary diffusing capacity values in the presence of extensive pulmonary disease.

Sumario

Utilidad Clínica de la Prueba de la Capacidad Difusora Pulmonar con Una Sola Respiración

La capacidad difusora pulmonar, conteniendo la respiración, (D/P) resultó estar atenida al tamaño del cuerpo y a la edad en un grupo de 135 enfermos sin mayor afección cardiopulmonar. Se han determinado las fórmulas de regresión para predecir la capacidad difusora pulmonar:

Para varones: $D_P = 15.5 \times$ área del cuerpo $- 0.238 \times$ edad $+ 6.8$;

Para mujeres: $D_P = 15.5 \times$ área del cuerpo $- 0.117 \times$ edad $+ 0.5$.

Se sugiere el concepto de una típica proporción entre la capacidad difusora pulmonar y el volumen pulmonar (D_P/V_P) y se ha determinado una fórmula de regresión aplicable a ambos sexos:

$$D_P/V_P = 6.49 - 0.0298 \times \text{edad}.$$

Aun tomando en cuenta edad, sexo y tamaño del cuerpo, existe una variabilidad muy notable de sujeto en sujeto en la capacidad difusora pulmonar y la capacidad difusora pulmonar/volumen pulmonar. Con ninguno de los parámetros estudiados pudo explicarse esta variabilidad.

Se presentan los resultados del estudio de la capacidad difusora en 555 enfermos. Estaba disminuída la capacidad difusora pulmonar en todos los trastornos cardiopulmonares estudiados si ellos iban acompañados de baja del volumen pulmonar y estaba reducida en el enfisema pulmonar. Sin embargo, no estaba alterada apreciablemente en el asma o la bronquitis sin enfisema concomitante.

Debido a la amplia variabilidad de los datos, es sumamente limitada la utilidad de la prueba de la capacidad difusora pulmonar, conteniendo el aliento, para determinar la existencia de enfermedad o para el diagnóstico diferencial. Puede poseer alguna utilidad para diferenciar el enfisema del asma o de la bronquitis, sobre todo si se considera la capacidad difusora pulmonar/volumen pulmonar, aunque no cabe considerar una baja capacidad difusora conteniendo el aliento como pauta necesaria para el diagnóstico de enfisema.

Se observó que una corriente alta de sangre pulmonar en una persona joven iba acompañada de algún aumento de la capacidad difusora pulmonar/volumen pulmonar, y esta prueba tal vez posea limitada utilidad en el diagnóstico de desviación de izquierda a derecha en las cardiopatías congénitas.

Se pone en duda la especificidad del término de síndrome de "bloqueo capilar-alveolar" para ningún grupo dado de enfermedades, como sucede también con la propiedad del término "bloqueo" para muchos de los sujetos en quienes se diagnostica este síndrome. El término de "funcionamiento inadecuado del parénquima pulmonar" parecería ser más aplicable generalmente.

Se confirma la excelente reproducibilidad de la

determinación de la capacidad difusora pulmonar, conteniendo el aliento, en pruebas sucesivas.

Se notó una correlación bien definida entre la capacidad difusora pulmonar y la insaturación arterial durante el ejercicio, pero hubo muchas discrepancias individuales. Por lo general, hubo una importante baja de la saturación de oxígeno durante el ejercicio con capacidades difusoras de menos de menos de 8 c.c. de monóxido de carbono por minuto por mm. de mercurio.

A base exclusivamente de su utilidad clínica, no parecen justificados el costo y las inconveniencias que acarrea la prueba de la capacidad difusora en una sola respiración, y hay que desplegar cautela en la interpretación de los valores de la capacidad difusora pulmonar con retención del aliento cuando existe extensa afección pulmonar.

Resume

Utilité clinique du test de la capacité de diffusion pulmonaire par une seule respiration

On a trouvé que la capacité pulmonaire de diffusion, (D_L), quand le sujet retient sa respiration, dépend de la taille du corps, de l'âge, dans un groupe de 135 malades sans maladie cardio-pulmonaire importante. On a déterminé des formules de regression pour prévoir la capacité pulmonaire de diffusion:

pour les hommes: $D_L = 15{,}5 \times$ surface corporelle $- 0{,}238 \times$ âge $+ 6{,}8$;

pour les femmes: $D_L = 15{,}5 \times$ surface corporelle $- 0{,}117 \times$ âge $+ 0{,}5$.

Le concept du rapport: capacité de diffusion pulmonaire caractéristique/volume pulmonaire (D_L/V_L) est proposé pour un âge donné, et une formule de regression valable pour les deux sexes a été déterminée:

$$D_L/V_L = 6{,}49 - 0{,}0298 \times \text{âge}.$$

Même quand l'âge, le sexe et la taille corporelle sont pris en considération, il y a une variabilité nette de la capacité de diffusion pulmonaire et du rapport capacité de diffusion pulmonaire/volume pulmonaire d'un sujet à l'autre. Cette variabilité ne peut pas être considérée par aucune des valeurs étudiées.

Le rapport porte sur les résultats sur la capacité de diffusion de 555 malades. La capacité de diffusion pulmonaire fut diminuée dans tous les troubles cardiopulmonaires étudiés, lorsqu'ils étaient accompagnés d'une chute du volume pulmonaire, et elle fut diminuée dans l'emphysème pulmonaire. Cependant la capacité de diffusion pulmonaire ne fut pas perturbée d'une façon appréciable dans l'asthme ou la bronchite sans asthme associé.

A cause de la grande variabilité des données, l'utilité du test de la capacité de diffusion pulmonaire est trés limitée pour déterminer la présence d'une maladie ou pour faire un diagnostic différentiel. Il peut avoir quelqu'utilité pour différencier l'emphysème de l'asthme ou de la bronchite, surtout si l'on considère le rapport capacité de diffusion pulmonaire/volume pulmonaire, bien qu'une faible capacité de diffusion à respiration retenue ne puisse certainement pas être considérée comme un critère nécessaire dans le diagnostic d'emphysème.

On trouva chez une jeune personne un débit sanguin pulmonaire élevé associé à quelqu'augmentation du rapport capacité de diffusion pulmonaire/volume du poumon et ce test peut avoir une utilité limitée dans le diagnostic de shunt gauche-droit dans les malformations cardiaques congénitales.

On met en question la spécificité du syndrôme du "bloc alvéolocapillaire" dans n'importe quel groupe particulier de maladies, comme est justifié le terme de "bloc" pour beaucoup de sujets chez qui on a diagnostiqué le syndrôme. Le terme "fonctionnement inadéquate du parenchyme pulmonaire" peut être appliqué d'une façon plus générale, semble-t-il.

On confirme la reproductibilité excellente de la détermination de la capacité de diffusion pulmonaire en retenant la respiration, dans les tests successifs.

On a noté une corrélation nette entre la capacité de diffusion pulmonaire et la non-saturation artérielle en exercice, mais beaucoup d'erreurs se produisirent. Une chute importante se produisit généralement dans la saturation d'oxygène en exercice, avec des capacités de diffusion de moins de 8 cc de monoxyde de carbone par minute par mm de mercure.

La dépense et le désavantage du test de la capacité de diffusion par une seule respiration ne semblent pas être basés seulement sur son utilité clinique, et on doit être prudent dans l'interprétation des valeurs de la capacité de diffusion pulmonaire en retenant la respiration, en présence d'une maladie étendue du poumon.

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SINGLE-BREATH PULMONARY DIFFUSING CAPACITY MEASUREMENTS IN PATIENTS WITH PULMONARY TUBERCULOSIS[1, 2]

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Introduction

Breath-holding diffusing capacity tests have been performed routinely by the pulmonary function laboratory of the Suburban Cook County Tuberculosis Hospital-Sanitarium on hospitalized patients referred for pulmonary function studies. Results on 221 patients studied to date are presented in order to assess the value of routine measurements of the diffusing capacity in evaluating the pulmonary function of patients with tuberculosis and to delineate those factors which influence the pulmonary diffusing capacity in patients with this disease.

Materials and Methods

The 221 patients consist of 144 men, ranging in age from eighteen to seventy-seven, and 77 women, ranging from seventeen to sixty-five. All but 5 were hospitalized at the time of testing, these 5 being outpatients who had formerly been hospitalized. This series consists of only those patients with diagnoses of tuberculosis. All patients in whom this disease was later ruled out have been excluded from the report. In most of the patients, the diagnoses were proved bacteriologically. However, a few cases are included in which tuberculosis was diagnosed from the clinical course, roentgenographic findings, and tuberculin status, although no bacteriologic confirmation of the diagnoses was obtained. The extent of disease was assessed as far advanced in 52 per cent, moderately advanced in 35 per cent, and minimal in 13 per cent of the patients. All of the patients tested were referred by staff physicians to the laboratory for pulmonary function tests, and no further selection was made for the purpose of this study. The 221 patients tested represent about 27 per cent of the hospitalized patient population over a two-year period (1959–1960).

No attempt was made to exclude patients with associated diseases or complications. Two patients had silicosis; 5, pleural effusions; 30, previous thoracic surgery (three or more months before the test); 15, asthma or asthmatic bronchitis; 18, a diagnosis of pulmonary emphysema; 2, empyema; 2, bronchopleural fistulas; and 2, bronchogenic carcinomas.

Routine pulmonary function studies were performed on all of the patients in this report, consisting of measurements of vital capacity, first-second vital capacity, maximal midexpiratory flow rate, maximal breathing capacity, residual volume, nitrogen washout curve, and carbon monoxide diffusing capacity (D_L). The residual volume was determined by a modification of the open-circuit method of Darling and co-workers (1), and ventilatory studies were performed on a standard 13.5-liter Collins Respirometer. The diffusing capacity was determined by Forster and associates' modification (2) of the single-breath test developed by Krogh. These diffusing capacities were compared with the values obtained by Burrows and associates (3) in a group of normal subjects in order to assess the amount of impairment of D_L in the patients with tuberculosis.

An attempt then was made to correlate the amount of impairment of diffusing capacity with the type and extent of pulmonary disease. Posteroanterior chest roentgenograms of the first 141 patients were reviewed by three physicians (two internists and one roentgenologist) in order to assess the extent and type of pulmonary disease. Noted particularly were the presence and extent of cavitation and cysts; and the disease was classified as localized, disseminated, fibrotic, or nodular, bilateral, or unilateral. The condition of the pleura, diaphragm, lung parenchyma, vasculature, ribs, and costophrenic angles was rated on a scale from 1 to 7 (1 was completely normal and 7 very extensively diseased). Also, an over-all general assessment of the extent of pulmonary disease of each of these patients was made, using the same scale. Evaluation of the roentgenograms was done without knowledge of the results of pulmonary function tests.

Results

Least-squares regression lines revealed a definite inverse relationship between D_L and age, as shown in figure 1*A* and *B*. The women showed only a slight decrease of D_L with increasing age—much less than the men—but the scatter is wide.

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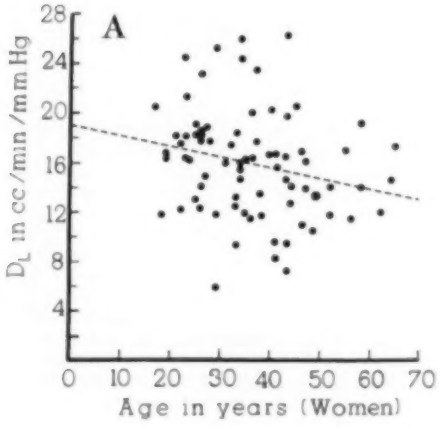
[2] Aided by a research grant from the Illinois Tuberculosis Association.

This is, in part, the result of wide lung volume variation at any given age, since lung volume also shows a definite correlation with D_L.

The relationship of carbon monoxide diffusing capacity to lung volume (V_L) is depicted in figure 2, using the thirty- to fifty-year age group. This limitation to a twenty-year span minimizes the scatter due to the effect of age on D_L. It may be seen that D_L correlates well with V_L, but that D_L increases less rapidly than V_L, and the least-squares regression line indicates a D_L of 6.2 at zero V_L.



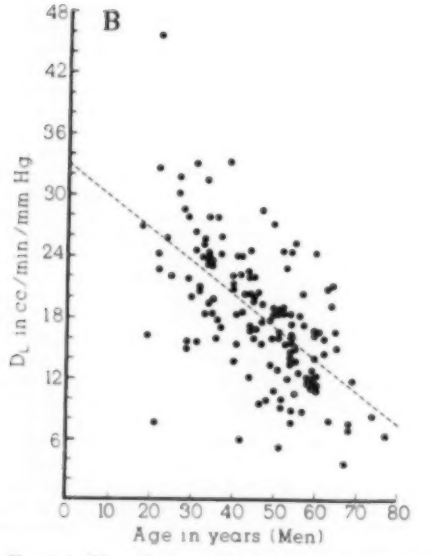


FIG. 1A (Upper). The relationship between diffusing capacity (D_L) and age in 77 female patients with pulmonary tuberculosis. The least-squares regression formula is $D_L = 18.95 - 0.0867 \times$ age. The standard error of estimate is 4.17, and the correlation coefficient, $r = -0.232$.

FIG. 1B (Lower). The relationship between D_L and age in 144 male patients with pulmonary tuberculosis. The least-squares regression formula is $D_L = 33.1 - 0.323 \times$ age. The standard error is 5.31, and $r = -0.608$.

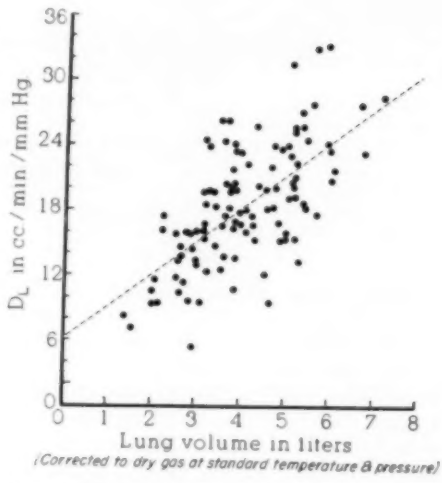


FIG. 2. The relationship between D_L and V_L in patients between the ages of thirty and fifty. The least-squares regression formula is $D_L = 6.28 + 3.02 \times V_L$.

In a group of tuberculous patients with wide variation in lung volume, a measurement of diffusing capacity independent of lung volume would be useful. The simplest measurement of this is the diffusing capacity (in cc. per min. per mm. of mercury) divided by the lung volume at the time of breath-holding (in liters of dry gas at standard temperature and pressure). This parameter, which is the diffusing capacity per liter of lung volume, hereafter will be called D_L/V_L, and while assumed to be independent of V_L, there is a slight tendency for patients with larger lung volumes to have lower D_L/V_L values (figure 2). The measurement of D_L/V_L would help to determine whether there was impairment of the diffusing characteristics of the aerated lung tissue such as that from a block to gas transfer across the alveolar-capillary membrane.

The effect of age on D_L/V_L is shown in figure 3*A* and *B*. The solid, steep line is the least-squares regression line for patients. The middle broken line is the least-squares regression line for

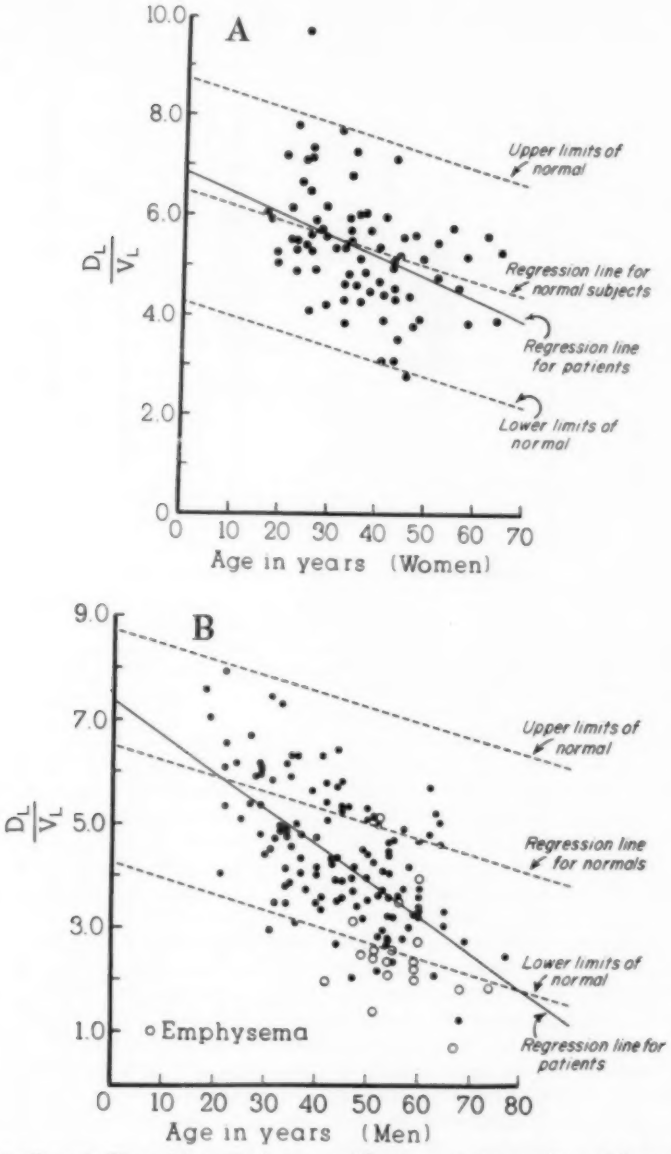


FIG. 3*A* (Upper). The relationship between diffusing capacity per liter of lung volume (D_L/V_L) and age in 77 female patients with pulmonary tuberculosis. The solid line is the least-squares regression line; its formula is: $D_L/V_L = 6.88 - 0.042 \times$ age. The standard error is 1.09, and $r = -0.407$. The middle broken line is the least-squares regression line of normal subjects, and the upper and lower broken lines denote two standard deviations from that line.

FIG. 3*B* (Lower). The relationship between D_L/V_L and age in 144 male patients with pulmonary tuberculosis. The least-squares regression formula is $D_L/V_L = 7.37 - 0.0694 \times$ age. The standard error of estimate is 1.09, and $r = -0.629$. Patients with clinically diagnosed emphysema are designated by a circle.

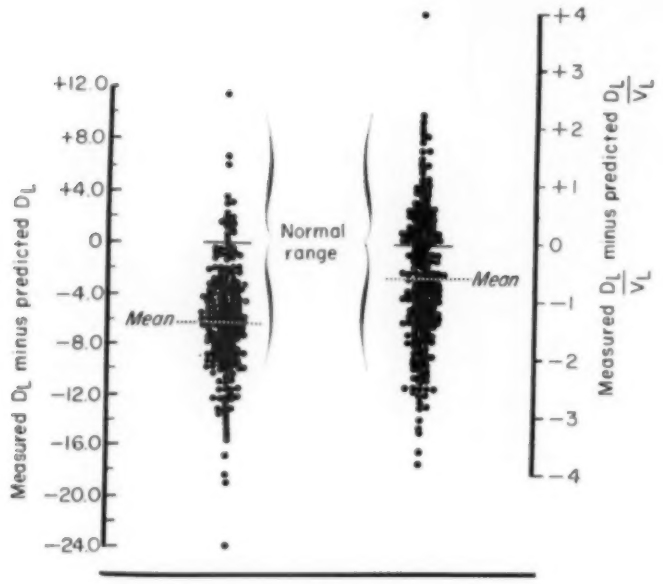


FIG. 4. The comparison of measured D_L/V_L minus predicted D_L/V_L with measured D_L minus predicted D_L in 221 patients with pulmonary tuberculosis. Plus values represent patients with measured D_L/V_L and D_L above predicted, and minus values D_L/V_L and D_L values below predicted. The normal range denotes two standard deviations about the mean of normal subjects' variation from predicted.

normal subjects,[8] and the upper and lower broken lines mark the normal range into which D_L/V_L measurements of 95% of the normal subjects fall. It is apparent that most of the values for D_L/V_L in female patients are within the normal range, and the regression line ($D_L/V_L = 6.88 - 0.042 \times$ age) is not significantly different from that of normal subjects. Most of the male patients less than forty years of age also have values for D_L/V_L which are within the normal range. However, male patients more than fifty years of age tend to have lower D_L/V_L values than normal subjects their age, and the least-squares regression line for the men is $D_L/V_L = 7.37 - 0.0694 \times$ age. This inverse relationship between age and D_L/V_L is significantly greater ($P = <0.01$ of chance occurrence) in the male patients than in normal men. Patients with clinical and physiologic evidence of pulmonary emphysema are designated by a circle in figure 3*B*, and it is evident that these patients tend to have reduced D_L/V_L values.

[8] The regression formula obtained from normal subjects is $D_L/V_L = 6.49 - 0.0298 \times$ age, and is used for both men and women (3).

A predicted D_L and D_L/V_L for each patient was calculated using the regression formulas of normal subjects. The discrepancy between predicted and measured D_L and between predicted and measured D_L/V_L for each patient is shown in figure 4. The normal limits represent two standard deviations from the mean of the normal subjects. It is apparent that measured values for the majority of patients are below the predicted and that some values are below the limits of normal. The average discrepancy between measured and predicted D_L is −6.2 cc. per min. per mm. of mercury, and that between measured and predicted D_L/V_L is −0.63 cc., fewer patients showing abnormally low values for D_L/V_L than for total D_L. The normal limits of variation from predicted D_L/V_L are ±2.24 cc. Twenty-two men and one woman have measured D_L/V_L values which are more than 2.24 cc. below their predicted D_L/V_L values. This group demonstrates an impairment of diffusing capacity out of proportion to the loss of lung volume.

The review of chest roentgenograms of the first 141 patients tested failed to reveal any parameters in addition to age, sex, and the pres-

ence of pulmonary emphysema which correlated significantly with reduced D_L/V_L. Thus, the presence of asthma, previous surgery, reduced vital capacity, or bilateral disease did not correlate at all with D_L/V_L. There was a slightly increased incidence of multiple cysts and of extensive cavitation in the group of patients with reduced D_L/V_L and a slightly higher extent-of-disease rating, as shown in table 1. Twenty-nine per cent of the patients with low D_L/V_L values had cavitation of a lobe or more in extent, while only 16 per cent of the patients with D_L/V_L values close to predicted and 11 per cent of the patients with better than predicted D_L/V_L values had such extensive cavitation. Multiple cysts also were more prevalent in patients with reduced D_L/V_L measurements. The average extent-of-disease rating (on the 1 to 7 scale) for the patients with low D_L/V_L values was 4.0, whereas it averaged 3.4 for the group of patients with normal and above-normal D_L/V_L values. These relationships, however, may merely have represented the presence of pulmonary emphysema and its effect on the roentgenographic appearance of the lungs. When patients with clinical diagnoses of emphysema were excluded, no definite differences were noted between the roentgenograms of patients with high, normal, or low D_L/V_L values.

TABLE 1

ROENTGENOGRAPHIC FINDINGS OBSERVED IN 141 TUBERCULOUS PATIENTS WHO WERE CLASSIFIED IN TERMS OF DIFFUSING CAPACITY PER LITER OF LUNG VOLUME (D_L/V_L) VALUES IN THE LOW, NORMAL, AND HIGH RANGES

	D_L/V_L Range		
	High*	Normal†	Low‡
Total number of patients	9	84	48
Per cent with far advanced disease	44	48	56
Per cent with bilateral disease	56	42	60
Per cent with cavitation of a lobe or more	11	16	29
Per cent with multiple cysts	0	7	12
Per cent with disseminated disease	44	48	52

* D_L/V_L more than one standard deviation (1.12 cc. per min. per mm. Hg) above normal

† D_L/V_L within one standard deviation of normal

‡ D_L/V_L more than one standard deviation below normal

DISCUSSION

In normal subjects, D_L/V_L has been found to be significantly influenced only by age. Lung volume appears to have a slight negative correlation with D_L/V_L in normal subjects, but this relationship is so slight and variable that it has been ignored in determining the formula for predicting D_L/V_L. Therefore, a uniform D_L/V_L ratio for a given age, regardless of the absolute value of V_L, has been assumed. Men have higher diffusing capacities than women, but D_L/V_L values of men do not seem to differ significantly from those of women subjects. Thus, while D_L is dependent on age, sex, body surface area, and lung size in normal subjects, D_L/V_L appears to be only age dependent (3, 4).

In patients with tuberculosis these same factors influence D_L and D_L/V_L. Although D_L is substantially reduced in many patients of all ages, much of this reduction may be accounted for by loss of lung volume rather than by a reduction of D_L/V_L. Therefore, in these cases the measurement of diffusing capacity adds little more information to that obtained by V_L determinations alone. However, there are a few patients in this study, particularly older men, in whom the D_L/V_L is reduced below normal limits.

Emphysema, which is known to be associated with reduced D_L and D_L/V_L (2, 3), is present in 16 (25 per cent) of the men more than fifty years of age in this series, and it is evident from figure 3*B* that emphysema has been clinically diagnosed in most of the patients with D_L/V_L values below the limits of normal. In addition, there is physiologic evidence that at least 3 of the remaining patients with reduced D_L/V_L values may have mild or early emphysema. Thus, in this series of 221 patients, as shown in figure 3*A* and *B*, there are only 6 nonemphysematous persons with D_L/V_L measurements which are below the limits of normal as they have been defined. This frequency is within the expected range when the normal limits are set at twice the standard deviation.

Even excluding patients with diagnosed emphysema, age has a much greater negative correlation with D_L and D_L/V_L in the men with tuberculosis than in normal males. When the D_L/V_L regression line is recalculated, excluding patients with diagnosed emphysema, it be-

comes: $D_L/V_L = 7.19 - 0.062 \times$ age. Although this relationship is less abnormal than the regression line based on the total group of patients, it still shows that the average D_L/V_L of male tuberculous patients more than fifty years of age is reduced. Attempts to explain this reduction by correlating the decreased D_L/V_L with type or extent of disease were not successful. On the basis of chest roentgenograms, it was impossible to separate patients with low D_L/V_L values from those with high values except in a few patients in whom emphysema was discernible. This conclusion was reached after attempts were made to estimate the measured D_L of each of 141 patients by review of their chest roentgenograms. Reasonable estimates could be made by accounting for the probable age and lung volume, as judged roentgenographically. However, when an attempt was made to judge if the D_L would be above or below average for the patient's age, sex, and lung volume, no separation of patients with more than average D_L values from ones with low values could be made at greater than chance level. Specific findings such as pleural involvement, extent and type of parenchymal disease, and previous surgery did not correlate at all with the degree of D_L/V_L impairment. Thus, it is impossible to define any specific roentgenographic pattern besides emphysema which tends to be associated with an unusually low D_L/V_L. The subnormal D_L/V_L measurements of older men with tuberculosis might be due to the development of subclinical and, perhaps, localized emphysematous change in response to their infection; at least, no other explanation is apparent.

The failure of extent or type of disease to correlate with D_L/V_L may be attributable to the wide variation of this parameter in normal subjects at any given age. Some of the tuberculous patients may have considerably reduced values for D_L/V_L from their pre-disease level, but this would not be detectable since normal averages have been used for comparison rather than pre-disease values. Furthermore, because of the wide limits of normal, it is impossible to tell whether the presence of pulmonary tuberculosis in older men has reduced the D_L/V_L values slightly in all of them, or greatly in some while sparing others. Either way, the average D_L/V_L of the patients would be reduced, but the meaning of the D_L/V_L determination in any individual would be questionable.

The lack of correlation of extent or type of disease with D_L/V_L and the minimal decrease in D_L/V_L in the absence of emphysema do not support the concept that alveolar-capillary block is characteristic of pulmonary tuberculosis. Rather, the loss of diffusing capacity would seem related to loss of functioning lung parenchyma with attendant reduction in diffusing surface and loss of functioning capillary bed. In this case, it is not surprising that resting D_L measurements fail to yield significant additional information to that obtained by ventilatory, nitrogen washout, and lung volume measurements.

Summary

Pulmonary diffusing capacity (D_L) measurements by the carbon monoxide breath-holding technique were performed on 221 patients with diagnoses of pulmonary tuberculosis, and the results were reviewed in order to assess the effect of tuberculosis on pulmonary diffusion. The diffusing capacity was found to correlate well with lung volume; since many patients had reduced lung volumes, their diffusing capacities also were reduced. The diffusing capacity per liter of lung volume (D_L/V_L) of most of the patients was within normal limits; the measurements were usually low in subjects with pulmonary emphysema, and male patients more than fifty years of age also tended to have lower than predicted values. Other factors, such as extent of disease, reduced vital capacity, or previous surgery could not be correlated with reduction of the diffusing capacity per liter of lung volume. It is concluded that routine single-breath diffusing capacity determinations in patients with pulmonary tuberculosis add little useful information to the data obtained from ventilatory and nitrogen-washout studies, and that "alveolar-capillary block" is not characteristic of any of the common forms of this disease.

Sumario

Mediciones de la Capacidad Difusora Pulmonar en Una Sola Respiración en Enfermos de Tuberculosis Pulmonar

En 221 enfermos en quienes se había diagnosticado tuberculosis pulmonar, se ejecutaron mediciones de la capacidad difusora pulmonar (D_P), repasándose el resultado para valorar el efecto de la tuberculosis sobre la difusión pulmonar. La capacidad difusora resultó correlacionarse bien

con el volumen pulmonar; como muchos enfermos tenían el volumen pulmonar reducido, también estaba reducida su capacidad difusora. La capacidad difusora por litro de volumen pulmonar (D_P/V_P) de la mayoría de los enfermos quedó dentro de límites normales; las mediciones fueron por lo general bajas en los sujetos que tenían enfisema pulmonar; y los varones de más de cincuenta años de edad también se inclinaban a mostrar cifras más bajas de las predichas. Otros factores, tales como la extensión de la enfermedad, la reducción de la capacidad vital o una operación anterior, no pudieron correlacionarse con la reducción de la capacidad difusora por litro de volumen pulmonar. Se deduce que las determinaciones rutineras de la capacidad difusora en una sola respiración en los enfermos de tuberculosis pulmonar agregan poca información útil a los datos obtenidos con los estudios de la ventilación y del arrastre de nitrógeno, y que el "bloqueo capilar-alveolar" no es típico de ninguna de las formas comunes de esta dolencia.

Resume

Mesures de la capacité de diffusion d'une seule respiration chez des malades atteints de tuberculose pulmonaire

On effectua des mesures de la capacité de diffusion pulmonaire (D_L), avec du monoxide de carbone, chez 221 malades ayant été diagnostiqués tuberculeux pulmonaires, en leur faisant retenir leur respiration, et on passa en revue les résultats de façon à établir l'effet de la tuberculose sur la diffusion pulmonaire. La capacité de diffusion fut, trouva-t-on, bien en rapport avec le volume pulmonaire; vu que beaucoup de malades avaient des volumes pulmonaires diminués leur capacité de diffusion fut aussi diminuée. La capacité de diffusion par litre de volume pulmonaire (D_L/V_L) fut dans les limites normales chez la plupart des malades; les mesures furent généralement basses chez les sujets ayant de l'emphysème pulmonaire, et les malades hommes au-dessus de cinquante ans eurent aussi tendance à avoir des valeurs inférieures à celles prévues. D'autres facteurs, tel que l'extension de la maladie, une diminution de la capacité vitale, ou une opération antérieure ne purent pas être rattachées à la diminution de la capacité de diffusion par litre de volume pulmonaire. On conclut que les déterminations habituelles de la capacité de diffusion par une seule respiration, chez des malades ayant une tuberculose pulmonaire, ajouta peu d'information utile aux données obtenues par les études de la ventilation et de l'expulsion de l'azote, et que "le bloc alvéolo-capillaire" n'est pas caractéristique d'aucune forme de cette maladie.

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THE DIFFUSING CAPACITY OF THE LUNG IN ACUTE PULMONARY TUBERCULOSIS[1, 2, 3]

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Introduction

Pulmonary tuberculosis may result in chronic impairment of lung function. This study was designed to develop and test a simple bedside method for the repeated measurement of the carbon monoxide diffusing capacity of the lungs in acutely ill, bedridden patients. It is hoped that such a method may prove to be a useful index of the progress of the disease. This report describes the method and its reproducibility and presents a comparison of estimates of abnormality based upon this test and upon posteroanterior and lateral roentgenograms of the chest.

Materials and Methods

The patients selected for this study were all adults with previously untreated tuberculosis who were less than fifty-one years old and who had no clinical evidence of airway obstruction. Since severe anemia may cause reduction of the diffusing capacity (1), patients in whom the hematocrit was less than 32 per cent were arbitrarily excluded from this study.

The diffusing capacity was measured by a modification of the breath-holding technique developed by Ogilvie and associates (2). A balloon filled with 0.3 per cent carbon monoxide, 4 per cent helium, 21 per cent oxygen, and 75 per cent nitrogen was brought to the patient's bedside together with rubber bags for collecting expired gas, stopwatch, clamps, noseclip, and a 3-way stopcock. The sitting patient was asked to empty his lungs completely and then to fill his lungs with the carbon monoxide-helium mixture. At the start of inspiration the stopwatch was started. During the period of breath-holding the patient's lips were connected to a 3-way stopcock and, after approximately ten seconds, he was instructed to breath out as rapidly and as deeply as possible. When a small (1-liter) rubber bag on the 3-way stopcock was filled, the cock was turned and the remainder of the expirate (the alveolar sample) was collected in an evacuated rubber bag for subsequent analysis. The stopwatch was stopped at the beginning of the collection of the alveolar sample. After a five-minute rest, the test was repeated. If the time of breath-holding was less than ten or more than fifteen seconds, the sample was discarded and the test repeated. If there was evidence of leak of air around the patient's mouth during inspiration or expiration, the gas was also discarded and the test repeated. The bags containing the alveolar samples were then taken to the laboratory for analysis of carbon monoxide, carbon dioxide, and helium contents, and the diffusing capacity was calculated from the conventional formula (2). The result was expressed as percent of normal on the basis of a formula derived from measurements of normal subjects in this laboratory (D_{CO} = Ht. $(CM/100)^3 \times 6$). The vital capacity was measured at the patient's bedside and the better of two expirations was used. The total lung capacity was assumed to be 1.25 times the measured vital capacity in the patients less than thirty-five and 1.31 times the vital capacity in the older patients (3).

Three observers estimated independently the percentage of pulmonary tissue not involved by tuberculosis on the posteroanterior and lateral films of the chest of each patient. This estimation was based on the assumption that the right upper lobe occupies 20 per cent of the lung; the right middle lobe, 10 per cent; the right lower lobe, 25 per cent; the left upper lobe, 20 per cent; and the left lower lobe, 25 per cent.

Results

As shown in table 1, the three observers independently rated the extent of roentgenographic involvement in each patient in a similar fashion. The Friedman two-way analysis of variance test on the ranked involvement for each patient yielded a X^2 value less than one. This indicates that no one of the observers

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[2] Presented before the medical session, as part of *Section* 9A, of the annual meeting of the American Thoracic Society, May 24, 1961.

[3] Supported by grants from the New York Tuberculosis and Health Association; the Health Research Council of the City of New York (Grant #U-1011) and grants #H-3838, #M-2572, and #A-2965 from the Division of General Medical Sciences, National Institutes of Health, U.S. Public Health Service.

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TABLE 1

IDENTIFYING DATA AND RESULTS OF MEASUREMENTS OF VITAL CAPACITY, OF DIFFUSING CAPACITY, AND OF INTERPRETATIONS OF THE ROENTGENOGRAMS OF 35 PATIENTS WITH TUBERCULOSIS*

Subject	Age	Sex	Height	VC† Per Cent Normal	Dco‡			Per Cent Normal	Roentgenograms			Mean
					Mean	Range	N		Per Cent Normal			
									Observer I	Observer II	Observer III	
			cm.		*cc./min./mm. Hg.*							
Edg.	45	M	175	40	9	7–10	3	28	65	62	70	66
Lon.	49	M	180	95	29	25–33	3	83	75	73	70	72
Doh.	18	F	160	68	19	18–20	2	76	90	90	90	90
Ort.	29	F	153	87	20	17–22	2	95	85	90	90	88
Mol.	34	M	175	75	22	21–23	2	69	85	80	75	80
Wil.	29	F	165	80	28	27–30	2	104	85	85	85	85
Orz.	50	M	165	81	21	16–27	2	78	80	83	75	80
Hor.	27	M	181	65	29	28–29	2	81	85	85	85	85
Mel.	15	F	147	70	20	17–24	3	105	65	80	65	75
Als.	38	M	180	48	17	17–18	2	49	80	75	80	78
Gri.	31	M	175	62	18	17–19	3	56	75	67	65	69
Vac.	26	M	183	69	24	23–25	2	65	75	70	75	73
Cla.	47	F	167	48	10	8–11	3	36	55	50	62	56
Clan.	36	F	167	48	7	7–8	3	25	35	60	50	48
Rod.	18	F	147	77	13	13–13	3	68	45	55	50	50
Rus.	38	M	180	57	22	17–28	3	63	70	70	70	70
Sed.	25	F	153	93	18	17–19	2	86	85	75	80	80
San.	22	F	155	77	13	13–13	4	59	70	65	70	68
Bar.	50	F	155	37	11	10–11	2	32	45	45	30	40
Edw.	28	F	165	62	15	15–15	2	56	80	80	80	80
Mar.	27	F	162	79	15	15–15	2	60	80	75	80	78
Her.	39	M	165	67	13	11–17	3	48	75	73	70	72
Dyr.	24	M	178	90	23	19–27	3	68	70	65	70	68
Jon.	36	F	173	69	18	17–18	2	58	80	85	80	82
Vas.	44	M	183	60	28	26–32	3	76	90	92	90	91
Rom.	22	M	173	92	20	18–23	4	64	80	70	80	77
Joh.	23	F	147	63	11	11–11	2	58	75	78	75	76
Doh. P.	16	M	183	62	30	30–30	2	81	80	84	80	81
Doh. J.	49	M	178	61	16	15–18	3	47	50	45	25	40
Hal.	42	F	175	58	17	16–19	3	53	55	62	55	57
Wil.	31	M	173	47	21	21–21	2	68	65	60	75	67
Red.	21	M	175	72	24	24–24	2	75	65	72	75	70
Bet.	37	M	173	56	12	12–12	3	61	65	72	70	69

* Vital capacity was expressed as per cent normal on the basis of the formula derived by Hepper and associates (5)

† Vital capacity

‡ Diffusing capacity

consistently rated the involvement low or high (4). Therefore, the arithmetic mean of the three estimates was used as the index of the percentage of normal lung on the roentgenogram for each patient. Analysis of the maximal deviations from the average for each patient revealed no significant differences from zero ($T = 1.30$). The maximal deviation from the mean for each patient, regardless of direction, averaged 4 per cent.

As shown in table 1, the technique for measurement of the diffusing capacity was found to yield highly reproducible data for the majority of patients. On the average, the first two observations on each patient differed by 2.3 cc. per min. per mm. of mercury, and the t value for

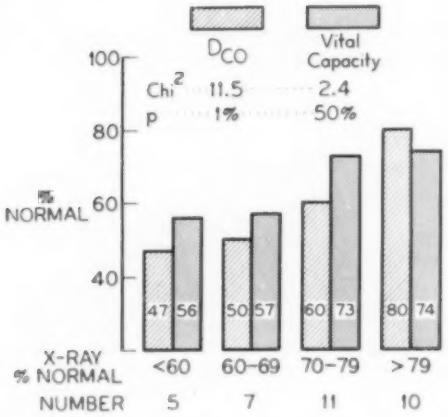

FIG. 1. Average values for diffusing capacity (D_{CO}) and vital capacity in 5 patients in whom less than 60 per cent of the lungs was judged to be normal roentgenographically, in 7 patients in whom 60 to 69 per cent of the lungs was normal, in 11 patients in whom 70 to 79 per cent of the lungs was normal, and in 10 patients in whom more than 79 per cent of the lungs was normal. The results of the chi-square tests indicated that there was a highly significant association between reduction of the diffusing capacity and the extent of roentgenographic involvement, but not between reduction of the vital capacity and the extent of roentgenographic involvement.

these differences was less than 1, indicating that there was no significant difference between the means of the two series. The mean diffusing capacity of each patient was calculated from the total number of analyses performed; and the average deviation from the mean, regardless of sign, for the entire series was only 1.3 cc. per min. per mm. of mercury.

The comparison of the measurements of diffusing capacity and of vital capacity with the roentgenographic findings is shown in figure 1. The association between reduction of vital capacity and roentgenographic involvement was poor. There was a highly significant association between the impairment of diffusion and the extent of roentgenographic involvement, indicating that the patients with the greatest amount of roentgenographic involvement had the greatest reduction of diffusing capacity, whereas those with the least amount of disease roentgenographically demonstrable had the highest diffusing capacities.

Discussion

The lack of correlation between the roentgenographic evaluations and the measurements of vital capacity is not particularly surprising. The vital capacity is dependent upon the integrity of the thoracic bellows, the chest wall, and the diaphragm; upon the ease with which the lung is stretched, the compliance; upon the amount of lung tissue which can be ventilated; and upon the presence of unobstructed airways. Although the chest film should provide a reflection of the amount of lung tissue involved by tuberculosis, it does not indicate the degree to which that tissue can be ventilated, nor does it provide information about the other factors which influence the vital capacity. Thus, the presence of parenchymal disease does not necessarily imply that ventilation is absent in the involved area or that adjacent areas may not be stretched abnormally so as to lead to preservation of a normal vital capacity. Conversely, the absence of parenchymal infiltration does not exclude the possibilities of airway obstruction, of restrictive pleurisy, or of poor use of the thoracic bellows, any one of which would lead to reduction of the vital capacity.

As the diffusing capacity of the lung depends upon the area of the pulmonary capillary bed as well as upon the thinness of the alveolar-capillary membrane, it might be expected that the amount of lung tissue involved by tuberculosis would be reflected in the degree to which the diffusing capacity was subnormal. In the present study, the correlation between impairment of diffusion and the extent of roentgenographic involvement was good, but the amount of impairment of diffusion appeared to exceed the amount of involvement demonstrated roentgenographically. It has long been known that pathologic examination generally reveals the presence of more extensive tuberculosis than is evident on the chest film, and the above data may indicate that the level of the diffusing capacity is actually a more accurate index of the extent of pulmonary tuberculosis than is the chest roentgenogram.

Summary

A method has been described for measuring the diffusing capacity of the lung at the bedside in patients acutely ill with tuberculosis. The

measurement is easy to perform and is highly reproducible. The association between impairment of diffusion and the extent of roentgenographic abnormality was good, whereas the association between reduction of the vital capacity and the extent of roentgenographic abnormality was poor. The data suggest that the diffusing capacity may be a more sensitive and accurate index of the extent of pathologic involvement by tuberculosis than the chest film.

Sumario

La Capacidad Difusora del Pulmón en la Tuberculosis Pulmonar Aguda

Se describe un método para medir la capacidad difusiva del pulmón a la cabecera del enfermo en sujetos agudamente enfermos de tuberculosis. La medición es fácil de ejecutar y sumamente reproducible. La asociación entre el menoscabo de la difusión y la extensión de la anomalía radiográfica fué buena, en tanto que fué mala entre la reducción de la capacidad vital y la extensión de la anomalía radiográfica. Los datos obtenidos sugieren que la capacidad difusiva puede ser un índice más sensible y exacto que la película torácica de la extensión de la invasión patológica por la tuberculosis.

Resume

La capacité de diffusion du poumon dans la tuberculose pulmonaire aiguë

Les auteurs ont décrit une méthode de mesure de la capacité de diffusion du poumon au chevet de malades atteints de tuberculose aiguë. La mesure est facile à faire et peut être tout à fait reproduite. Il y eut une bonne corrélation entre la diminution de diffusion et l'étendue des anomalies radiologiques, alors que la correlation entre la réduction de la capacité vitale et l'étendue des anomalies radiologiques fut de pauvre qualité. Les données font penser que la capacité de diffusion peut être un index plus sensible et plus fidèle de l'étendue de l'envahissement pathologique tuberculeux qu'une radiographie thoracique.

Acknowledgment

The assistance of Dr. Bernadette Drolette, who performed the statistical analyses, is gratefully acknowledged.

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MULTIPLICATION OF MYCOBACTERIUM TUBERCULOSIS IN THE LUNGS AND ITS MODIFICATION BY IMMUNITY AND BY ISONIAZID[1]

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INTRODUCTION

It has been shown previously (1) that, when *M. tuberculosis* is injected into the corneas of mice there is, at first, no spread of the infection to other organs. After a few months, however, systemic spread does occur, and large tuberculous lesions ultimately develop in the lungs. When such mice receive a challenge inoculum of *M. tuberculosis* into the cornea of the other eye, there is no multiplication of the organisms, which is presumably due to the effect of immunity. Nevertheless, organisms in the lungs are able to multiply and thus seemingly escape the effect of immunity.

These results strongly suggest that there are differences between the effect of immunity in the cornea and in the lungs, respectively. For this reason it seemed desirable to study the multiplication of *M. tuberculosis* in the lungs and the effect of immunity on this. At the same time the effect of isoniazid was also investigated, in comparison with a previous quantitative study on the effect of immunity and of isoniazid on the multiplication of *M. tuberculosis* in the cornea (2).

METHODS

The infection was initiated by direct inoculation into the lung, the method being devised in order to inject a known number of mycobacteria into the organ. This was done as follows: The hair was clipped along one side from under the armpit to the hip, using a Clukes clipper. The area was swabbed with alcohol and, under ether anesthesia, an incision was made in the lateral line below the armpit. The skin was retracted on both sides, the subjacent tissue was then divided and similarly retracted. With a good light, a lobe of the lung could then clearly be seen between the ribs through the intercostal muscles. Using a tuberculin syringe with a fine needle, 0.02 ml. of an undiluted culture of *M. tuberculosis* var. *bovis* in Dubos medium was injected through the intercostal muscles into the lung. The skin incision was then closed and the mouse was placed in a warm box where it rapidly recovered, and the mortality rate was low. Control studies with an injection of india ink confirmed that the inoculum was confined to a single lobe of the lung. Although the infection was thus initiated in one lobe of one lung, a preliminary investigation showed that it rapidly spread to both lungs; hence, more reliable results would be obtained if counts were made in all the lung tissue. Both lungs were therefore removed for counts in all cases. For each estimation of both culturable and total counts, the mean of the results obtained from the lungs of two mice was used.

At various periods after inoculation the mice were killed by breaking the neck, and the whole animal was dipped for a few seconds in absolute alcohol. The lungs were then removed with aseptic precautions and placed in 2 ml. of a mixture of collagenase and hyaluronidase, similar to that used by Robson and Smith (3) for the digestion of the cornea. After incubation at 37°C. for approximately eighteen hours, the tubes were shaken for thirty minutes on a microid flask shaker. The material so obtained was used for the determination of both the total counts and the counts of culturable mycobacteria. In order to prevent the multiplication of contaminating organisms during the digestion procedure, 5 units of penicillin and 0.01 mg. of chlortetracycline were added to each ml. of the collagenase/hyaluronidase solution. Control tests have shown that these agents have no significant effect on the mycobacteria under the conditions of these experiments (4).

The number of culturable organisms was determined by the method of O'Hea (5) in which Löwenstein-Jensen medium, which contains malachite green, is used. This seemed desirable in order to inhibit the growth of contaminating organisms likely to be found in the lungs. This method has been compared with that of Knox (6) and has been found to give similar counts (4). The total number of mycobacteria was then determined by the method previously described by Robson and Smith (3) and Smith (4).

Mice were vaccinated by the intradermal injection of 0.01 ml. of a 10^{-6} dilution of a culture of *M. tuberculosis* var. *bovis* in Dubos medium, as previously described by Acharya, Robson, and Sullivan (7).

When mice were treated with isoniazid, this was administered in the diet, 0.3 mg. of the drug

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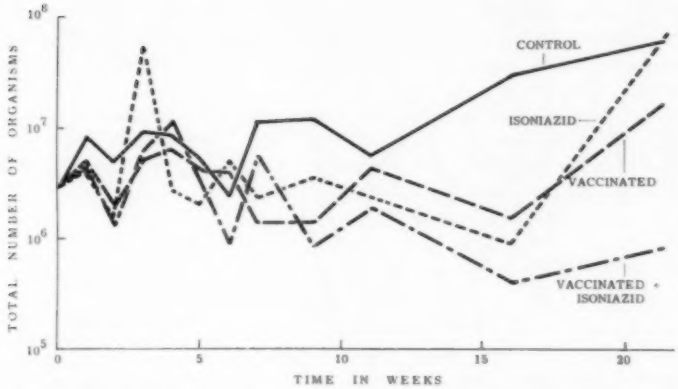

FIG. 1. Showing the effect of previous vaccination and of isoniazid treatment on the total number of mycobacteria in lungs of mice inoculated directly into the lung with a culture of *M. tuberculosis* var. *bovis*.

being contained in 5 gm. of Medical Research Council diet 41*B*, which is approximately the daily food intake of each mouse. This gives a dose of about 15 mg. per kg.

The experiment was performed on 4 groups of 30 mice each, two of which (*Groups 2* and *4*) had been vaccinated (as described above) one month previously. All of these mice were inoculated directly in the lung as described above. The groups were as follows:

Group 1. Nonimmunized, untreated (control)
Group 2. Immunized, untreated (vaccinated)
Group 3. Nonimmunized, treated with isoniazid from the time of infection until the end of the experiment (isoniazid)
Group 4. Immunized, treated with isoniazid from the time of intrapulmonary challenge until the end of the experiment (vaccinated and isoniazid).

RESULTS

The results are shown in figures 1 and 2. It will be seen that there are no striking differences between the four groups studied, with regard to both the total and the culturable counts. This is very different from the results in the cornea, in which large differences between some of the groups studied were observed (2). In order to detect small differences, the present data were analyzed statistically, and the results obtained for the total and culturable counts, respectively, are shown in tables 1 and 2. The statistical analyses were carried out on all of the values obtained, logarithms (to the base 10) being used. Obviously, there are no significant differences between the total counts for the various groups. However, the culturable counts for all three groups receiving some treatment were significantly different from those of the control. It may thus be concluded that both vaccination and isoniazid (as well as a combination of the two) produced a decrease in the number of culturable mycobacteria in the lungs.

The results, taken at their face value, appear to show that little or no multiplication of the mycobacteria occurred in the lungs even in the control group, in contrast to the cornea in which the number of organisms in the control mice increased by at least some fifteen times during the first two weeks after inoculation (2). It is, however, possible that such multiplication did occur in the lungs and was masked by a simultaneous clearance of organisms from the lungs. In order to examine this possibility, two experiments were performed.

Experiment 1: In the first experiment, mice were inoculated in the lung and were subjected to intensive chemotherapy which would, in all probability, prevent any multiplication and thus reveal any clearance, should this occur. Twelve mice were inoculated directly in the lung with 0.02 ml. of an undiluted culture of *M. tuberculosis* var. *bovis* in Dubos medium and treated with a combination of streptomycin (200 mg. per kg. per day, given in a single subcutaneous injection) and isoniazid (3.0 mg. per mouse per day, equivalent to about 150 mg. per kg., given in the food). This treatment was continued during the whole period of investigation.

The results for total and culturable counts

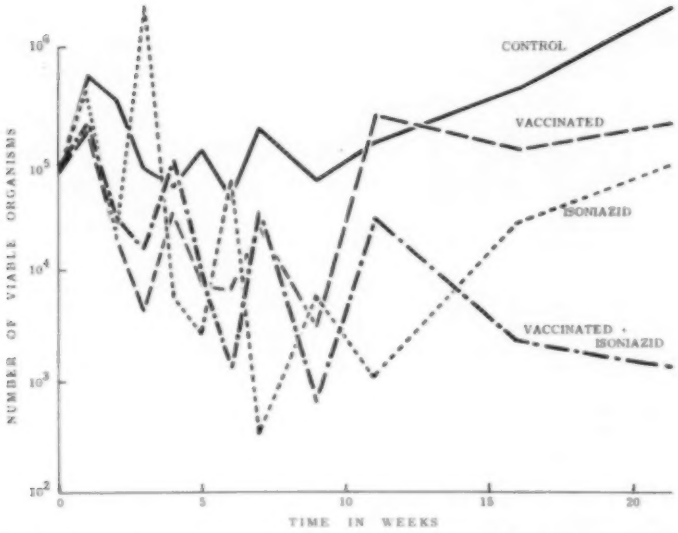

FIG. 2. Showing the effect of previous vaccination and of isoniazid treatment on the number of viable mycobacteria in lungs of mice inoculated directly into the lungs with a culture of *M. tuberculosis* var. *bovis*.

TABLE 1
STATISTICAL ANALYSIS OF DATA SHOWN IN FIGURE 1

Groups	*t*-value	Probability
1 and 2	0.3145	0.8
1 and 3	0.6276	0.6
1 and 4	1.138	0.3
2 and 3	0.2954	0.9
2 and 4	1.494	0.2
3 and 4	0.8590	0.4

TABLE 2
STATISTICAL ANALYSIS OF DATA SHOWN IN FIGURE 2

Groups	*t*-value	Probability
1 and 2	2.818	0.01
1 and 3	3.990	0.001
1 and 4	5.505	0.001
2 and 3	0.812	0.5
2 and 4	1.629	0.2
3 and 4	0.9435	0.4

are shown in figure 3. It will be seen that a marked fall in the total count occurred during the first two days following inoculation, after which the count became stable. The count of culturable organisms, however, continued to fall, showing how much more effective the intensive chemotherapy was (than a lower dose of isoniazid alone, *see* figure 2) in killing off the mycobacteria. These results do not exclude the possibility that the marked fall in total count is due to a loss in acid-fast staining, although the recent results of Rees and Hart (8) strongly

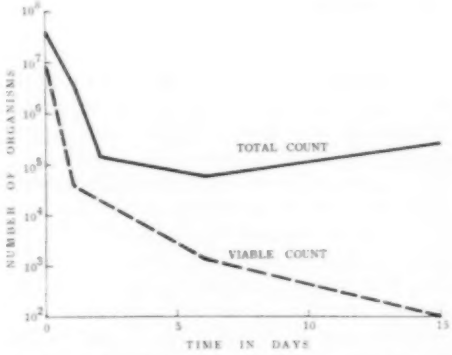

FIG. 3. Showing the fall in the total and culturable mycobacterial counts which occurs in the lungs of mice inoculated directly into the lung with *M. tuberculosis* var. *bovis* and receiving intensive chemotherapy.

suggest that intensive and prolonged chemotherapy (with isoniazid and pyrazinamide in that case) does not abolish the acid-fast staining of *M. tuberculosis*.

An additional experiment was performed to check this point (particularly as the results of Rees and Hart (8) were not known to the writers at that time).

Experiment II: Animals were inoculated with a nonmultiplying strain of *M. tuberculosis* (H37Ra) and no treatment was given. In this way it was expected that any fall in total count observed would be due to clearance of the organisms. Twelve mice were inoculated directly in the lung with 0.02 ml. of an undiluted culture of *M. tuberculosis* (H37Ra) in Dubos medium, and total counts were performed.

The results (figure 4) are in agreement with those obtained with the virulent bovine strain subjected to intensive chemotherapy in showing that a marked fall in total count occurred in the lungs during the first two days after inoculation. There is thus good reason for believing than when *M. tuberculosis* is injected directly into the lung, more than 90 per cent of the organisms are cleared from this organ within the first few days after inoculation. Support for the view that marked clearance occurred during that time is provided by the finding of many mycobacteria in various organs, namely, liver, spleen, and axillary lymph nodes, in the animals inoculated in the lung in the main experiment. Furthermore, mycobacteria were no longer found in these various organs at a later period, in agreement with the idea that clearance had then ceased.

In order to determine whether the pulmonary tuberculosis had stimulated "intracorneal" immunity, the remaining mice in each group were challenged intracorneally with a 10^{-3} dilution of a culture of *M. tuberculosis*; at the same time, a group of control mice was similarly inoculated. The latter all developed corneal lesions in the expected manner, while no lesions developed in any of the four groups of mice that had the pulmonary infection. Hence, it is evident that immunity, as judged by intracorneal challenge, was present in all of the mice with pulmonary tuberculosis.

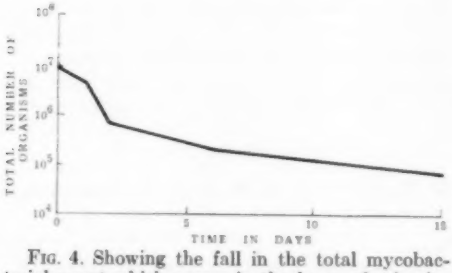

FIG. 4. Showing the fall in the total mycobacterial count which occurs in the lungs of mice injected directly into the lung with H37Ra.

DISCUSSION

The main purpose of this investigation was to resolve the following paradox: Some months after intracorneal inoculation of *M. tuberculosis*, large lesions tended to develop in the lungs of the mice; yet at this time sufficient immunity was present to prevent any appreciable mycobacterial multiplication, with the production of even the smallest macroscopic lesion, when a challenge inoculum was deposited into the other cornea (1). This strongly suggests that there is some important difference in the effect of immunity in the cornea and lungs, respectively. The results obtained in the present investigation do in fact support this hypothesis. In the cornea, as shown in previous experiments, immunity caused a rapid decrease in the count of culturable tubercle bacilli and ultimately very few live organisms could be recovered from that tissue (2). In the lungs, on the other hand, the fall due to immunity was much smaller and its significance only became definite on statistical analysis. Moreover, at the end of the experiment, the number of culturable organisms in the lungs of the vaccinated mice was not much smaller than that in control animals (*see* figure 2).

There appears to be another difference between the results obtained in the cornea and in the lungs. In the cornea, rapid multiplication of organisms was seen during the first fortnight after inoculation, involving both the total and culturable counts (2). At first sight, the curves (*see* figures 1 and 2) of the present experiments originally suggested that this did not occur in the lungs. However, the finding that there was a marked clearance of the mycobacteria from the lungs immediately after inoculation (*see* figures 3 and 4) shows that this conclusion was erroneous and that a good deal of multiplication must have occurred in the lungs during the first week of infection, thus keeping the bacterial population constant.

It is interesting that the various treatments used had no effect on the total counts in the lungs, although they had a significant effect on the counts of culturable organisms. As the organisms did in fact multiply during the first week after infection and did not appreciably increase in number after this time (as seen by the total count), it seems that the treatments used had no effect on the disease during the first week. This is in contrast to the finding in the cornea, in which the same treatments had a marked effect on bacterial multiplication during the first week (2).

After this time, however, treatment must have produced some effect in the pulmonary tuberculosis, since the culturable counts for the lungs were lower in all three treated groups than in the controls. It thus appears that in tuberculous infection of the mouse lungs, treatment takes effect only after a latent period of some days. This is indeed in keeping with the results of Niffeneger and Youmans (9) who found that, when mice were vaccinated with BCG and subsequently challenged with H37Rv, multiplication of the mycobacteria in the lungs proceeded as in control animals for at least a week, after which the multiplication was checked by the effect of immunity. Similarly, Walter and associates (10) found that the effect of isoniazid in tuberculosis in mice took effect only after a latent period of about a week, as shown by both the culturable counts (whole body homogenates) and by the body weight of mice infected with H37Rv.

The infection studied here was obviously chronic rather than acute, since no mice died during the experiment. It is interesting in this connection that Hart and Rees (11) found that the total and viable counts remained stable in a chronic lung infection in mice produced by inoculation with H37Rv. Moreover, they also showed that macrocyclon had no effect on such an infection, whereas, in an acute infection, the drug slowed down the multiplication of the mycobacteria in the lungs and prevented the early fatal outcome seen in untreated animals. It is well known that actively multiplying organisms are particularly susceptible to adverse conditions (12) and also that isoniazid is relatively ineffective for mycobacteria in the resting stage (13).

Thus it seems that the relatively small effects produced by immunization, drug treatment, and a combination of the two on the disease in the lung may merely reflect its chronic nature, though it is puzzling also that the immunity so produced was sufficient to prevent any bacterial multiplication in an inoculum injected into the cornea, i.e., when these mice were inoculated intracorneally at the end of the experiment.

Finally, these results do show that the action of immunity in the cornea and lungs differs in the important respect that in the one tissue there is a marked bactericidal effect which is not observed in the other. The cause of this difference is at present unknown, but it may well be one of the important factors explaining the different course of tuberculosis in different organs of the body.

Summary

The multiplication of *M. tuberculosis* var. *bovis* in the lungs of mice was determined by counting the total number and the number of culturable tubercle bacilli released by enzymatic digestion of the lungs. The effect of immunity, isoniazid treatment, and a combination of the two was studied.

In all groups the total bacterial population showed no great change throughout the experiment. However, it was shown that more than 90 per cent of the bacteria were cleared from the lungs during the first few days after inoculation; hence, multiplication, which made up the loss, must have taken place.

Vaccination, isoniazid (15 mg. per kg.), and a combination of both thus had no effect on the total bacterial population. These treatments did, however, have a significant effect on the counts of culturable organisms.

It is concluded that the various treatments have no effect on the early stages of the disease, but subsequently produce a decrease in the number of living mycobacteria in the lungs.

These results are compared with those obtained previously in tuberculosis produced experimentally in the cornea, in which immunity had a much more rapid and striking effect.

Sumario

La Multiplicación del Mycobacterium Tuberculosis en los Pulmones y Su Modificación por la Inmunidad y por la Isoniacida

Se determinó la multiplicación del *M. tuberculosis* var. *bovis* en los pulmones de ratones obten-

iendo numeraciones totales y culturables en las bacterias liberadas por la digestión encimática de los pulmones. Se estudió también el efecto de la inmunidad, la isoniacidoterapia y una combinación de ambas.

En ninguno de los grupos, mostró la población bacteriana total mayor alteración durante el tratamiento. No obstante, se observó que más de 90 por ciento de las bacterias fué eliminado de los pulmones durante los primeros días consecutivos a la inoculación; a partir de ahí, debe haber tenido lugar multiplicación, que compensó la pérdida.

La vacunación, la isoniacida (15 mg. por kg. de peso) y una combinación de ambas no habían, pues, ejercido efecto sobre la población bacteriana total; esos tratamientos ejercieron sí efecto importante sobre la numeración de bacterias culturables.

Se deduce que los varios tratamientos no ejercen efecto sobre los períodos tempranos de la enfermedad, pero más tarde producen una disminución en el número de bacterias vivas en el pulmón.

Se comparan estos resultados con los obtenidos anteriormente en la tuberculosis producida experimentalmente en la córnea, en la que la inmunidad ejerció un efecto mucho más rápido y notable.

Resume

Multiplication de Mycobacterium tuberculosis dans les poumons et sa modification par l'immunité et l'isoniazide

On détermina la multiplication de *M. tuberculosis*, var. *bovis*, dans les poumons de souris en obtenant les comptes totaux et cultivables des bactéries libérées par la digestion enzymatique des poumons. On étudia l'effet de l'immunité, du traitement par l'isoniazide et par l'association des deux.

Dans tous les groupes, la population totale bactérienne ne montra pas de grand changement pendant l'expérimentation. Cependant, on démontra que plus de 90% des bactéries furent éliminées des poumons pendant les quelques premiers jours après l'inoculation; par conséquent, une multiplication, pour compenser la perte, a du avoir lieu.

La vaccination, l'isoniazide (15 mg par kg) et une association des deux n'eurent ainsi pas d'effet sur la population totale bactérienne; ces traitements eurent certes cependant un effet important sur les comptes cultivables.

On conclut que les divers traitements n'eurent pas d'effet sur les stades précoces de la maladie, mais produisirent par la suite une diminution du nombre des mycobactéries vivant dans les poumons.

Ces résultats sont comparés à ceux obtenus antérieurement dans la tuberculose provoquée expérimentalement dans la cornée, dans laquelle l'immunité eut un effet beaucoup plus rapide et plus frappant.

Acknowledgment

The writers are grateful to the Colonial Medical Research Committee for a personal grant (to J.T.S.).

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INTERMITTENT ISONIAZID THERAPY FOR MICE[1, 2]

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Introduction

It is generally recognized that mass treatment schemes in tuberculosis campaigns will have to make use of ambulatory chemotherapy. This is especially true in areas with large numbers of untreated patients and inadequate hospital facilities. A recent study from the Tuberculosis Chemotherapy Centre in Madras, India (1), showed that the therapeutic results obtained in two carefully controlled comparable groups of patients were equally good in hospitalized and in nonhospitalized subjects. One of the difficulties encountered in implementing home-treatment schemes successfully is the fact that there is no reliance on the regularity with which patients are taking their drugs. It would therefore be advantageous if regimens could be devised which would permit drug intake at less frequent intervals without affecting the therapeutic results. For example, if drugs taken only once a week were equally effective as when taken three times a day, it might be possible to supervise the patients' weekly drug intake and thus ensure regular regimens in ambulatory patients.

With this practical approach in mind, the following animal experiments were undertaken. As will be shown, intermittent treatment with isoniazid was comparable in its effects to continuous regimens. It is recognized that results obtained in mice cannot readily be transferred to human patients. The answer to whether there is useful information to be gained from these experiments must come from clinical trials, but it is hoped that the present results may be regarded as sufficiently encouraging to stimulate similar work in patients.

Materials and Methods

Animals: Male CF_1, mice, weighing between 20 and 25 gm. at the onset of the experiment, were obtained from Carworth Farms, New City, New York. They were kept in groups of 10 and fed a powdered pellet diet and water ad libitum. The powdered diet was given in compartmentalized spill-proof feeders. All animals were weighed twice weekly in groups.

Infection: Mice were infected intravenously with bacteria from 2-week-old cultures of *M. tuberculosis*, strain Ravenel, propagated in liquid Tween®-albumin (2). The standard infective dose consisted of 0.2 ml. of a culture adjusted to an optical density of 0.1, as measured in a Coleman Junior spectrophotometer at $\lambda = 550$. Such suspensions contained approximately 4×10^7 viable units per ml. After infection, the mice were randomly distributed in experimental groups. The average survival time of untreated mice infected with this dose of bacilli was seventeen to twenty days.

Chemotherapy: Only isoniazid was used. The mice were either fed isoniazid with a stomach tube, the desired amount of drug being dissolved in 0.2 ml. of water, or isoniazid was mixed with the powdered diet and the medicated diets were put into and removed from the cages at fixed hours of the day. The daily food intake of a healthy mouse was about 4 gm.

Bacterial cultures from organs: Three randomly selected mice from a group were sacrificed at intervals as indicated, and lungs and spleens were removed under aseptic conditions. The organs were ground with a Teflon grinder in Pyrex glass tubes (3). Each organ was homogenized in 10 ml. of saline, containing 0.05 per cent bovine serum albumin and 1,000 units of penicillin per ml. The homogenate was incubated for two hours at 37°C., a treatment which killed most contaminants without affecting the tubercle bacilli. After incubation, serial tenfold dilutions were made in saline/albumin (without penicillin). From each dilution tube, Löwenstein-Jensen plates in 50-mm. Petri dishes were inoculated in triplicate with 0.5 ml. of suspension each. The colonies were counted after 4 weeks' incubation at 37°C. The dilutions yielding between 9 and 90 colonies per plate were taken to calculate the total number of viable units per organ. Thus, the average values for each point given in the protocols below were obtained from a total of 9 plates (triplicate platings of one dilution step for one organ each from 3 mice). Since each organ was homogenized in 10 ml. of diluent and 0.5 ml. of this homogenate was plated out, the theoretic limit of sensitivity of the method was 20 viable bacterial units per organ.

Drug-susceptibility testing: Isoniazid resistance was measured by the indirect method, using

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Löwenstein-Jensen slants containing isoniazid in the following concentrations: 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, 100.0 γ per ml. After four and six weeks' incubation, the tubes were inspected and the lowest concentration of isoniazid which inhibited growth completely was determined. The colonies were counted when there were fewer than 20 on a slant. When growth was more abundant, its intensity was estimated and recorded as + to +++. All determinations were done in duplicate. In some experiments, the emergence of drug resistance was also followed by the method of Tirunarayanan and Vischer (4).

The presence of catalase activity was estimated in aliquots of the same bacterial suspensions which served to inoculate the isoniazid-containing tubes. Using Wassermann tubes, 0.25 ml. of a 30 per cent hydrogen peroxide solution and 0.25 ml. of a 10 per cent solution of Tween 80 in water were mixed with 0.5 ml. of the bacterial suspension and left at room temperature for a few minutes. Presently, the tubes containing catalase-positive organisms showed a high "collar" of foam which was absent in tubes with catalase-negative organisms. The intensity of the oxygen production was estimated in an arbitrary scale ranging from 0 to +++.

Determination of isoniazid concentrations in mouse serum: Drug concentrations in mouse serum were determined biologically. Mice were bled from the retro-orbital plexus, the blood from 3 to 5 animals was pooled, and the serum was separated, inactivated, and filtered through millipore filters. Serial dilutions of these serum specimens were made in saline, and 0.1 ml. of the serum dilutions was added to tubes containing 1.0 ml. of oleic-acid-albumin medium (2). The tubes were inoculated with 1 drop from a calibrated Pasteur pipette of a 1:10 dilution of a 2-week-old culture of *M. tuberculosis*, strain Ravenel, in Tween albumin medium. Visible growth was recorded and inspected microscopically after three, five, and seven days. Isoniazid was titrated at the same time in comparable growth tubes. Control experiments showed that the effect of isoniazid was uninfluenced by the presence of mouse serum in any concentration up to 10 per cent, which was the highest serum concentration used in this testing procedure. Five-hundredths of a microgram of isoniazid per ml. gave complete growth inhibition under these conditions. The amount of active isoniazid in the serum was calculated from the isoniazid titration carried out at the same time.

Results

Experiment 1: Eighty-four mice were infected with a standard dose of *M. tuberculosis*, strain Ravenel. Four days after infection, they were randomly distributed in seven groups of 12 mice each and treated as follows:

Group I received 0.1 per cent of isoniazid in the diet for 21 consecutive days.

Group II received 0.01 per cent of isoniazid in the diet for 21 consecutive days.

Group III received 0.01 per cent of isoniazid in the diet on alternating days for a total of 21 days. Thus, during 3 weeks, these mice were alternatingly on and off isoniazid for 24-hour periods.

Group IV received 0.1 per cent of isoniazid in the diet twice a week (Mondays and Thursdays) for 24 hours for 3 weeks. Thus, these mice were given isoniazid in their diet for a total of 6×24 hours.

Group V received 0.01 per cent of isoniazid in the diet for 2 consecutive days (Mondays and Tuesdays) each week during 3 weeks. The mice were thus given isoniazid for a total of 3×48 hours.

Group VI received 0.1 per cent of isoniazid in their diet for one day each week (Mondays) for 3 weeks. Thus, these mice had isoniazid for a total of 3×24 hours.

Group VII received no isoniazid.

On the twenty-fifth day after infection, i.e., 3 weeks after the first day of treatment, all mice were put on a standard nonmedicated pellet diet. The days of death were recorded and all dead mice were autopsied to ascertain the presence of tuberculous lesions. The experiment was discontinued 158 days after infection.

The survival of these mice is shown in figure 1. As seen from this figure, there was a fair, although not complete correlation between the total intake of isoniazid during the three weeks following the fourth day after infection and the survival of the mice. In mice, the length of survival after tuberculous infection is a direct function of the number of infecting organisms (6). It may therefore be concluded that, with each of the various drug regimens, a certain proportion of the injected microorganisms was destroyed by isoniazid and that the length of survival subsequent to the cessation of therapy was proportional to the number of the surviving bacteria.

An approximate impression of the bactericidal effectiveness of the drug action is given in figure 2. It illustrates the results of an experiment in which a group of 10 mice received the standard dose of tubercle bacilli, a second group one half of this dose, and a third, one fourth of it. By extrapolation, it may be concluded that, in *Experiment 1*, even the weakest dose of isoniazid must have reduced the number of viable tubercle bacilli to approximately 20 per cent of the original inoculum. *Experiment 1* was discontinued after 158 days when its trend became clear: The mice of Groups II to V lost weight and were probably going to die. On the other hand, no weight loss had yet occurred in Group I. The average weight of these animals climbed steadily from an average of 20.8 gm. at the beginning of the experiment to 30.2 gm. at the end. At autopsy,

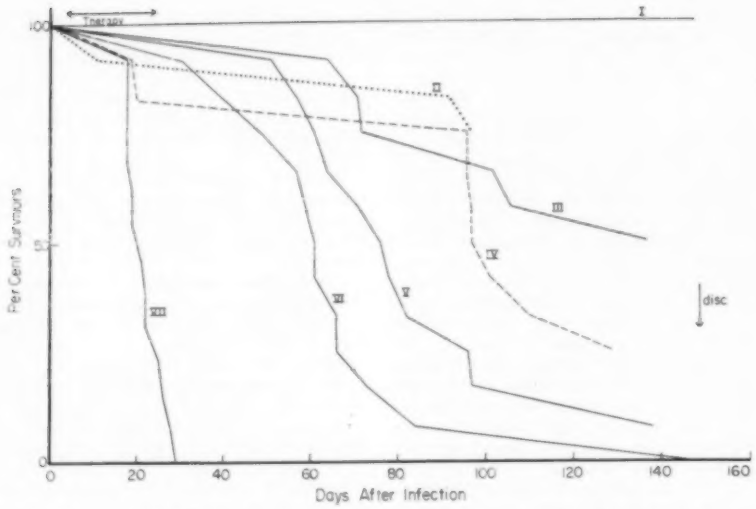

FIG. 1. The survival of tuberculosus mice after cessation for 3 weeks of various regimens of isoniazid. (*See* text for details.)

the sacrificed mice showed very extensive pulmonary disease with pneumonic consolidation, as is characteristically seen in chronic murine tuberculosis. No gross differences could be seen between the survivors of any of the groups, except for 3 mice in Group I which had distinctly smaller lesions and more remaining normal lung tissue than the rest of the animals in this and other groups.

Experiment 2: One hundred and sixty-six mice were infected with a standard dose of *M. tuberculosis*, strain Ravenel. Four days later, they were randomly distributed in cages and divided into four experimental groups. Except for one group of 10 mice which served as infection controls, the remaining three groups of 52 mice each (labeled *A*, *B*, and *C*) were now given 0.05 per cent of isoniazid in the diet for 7 days. After this initial period of uninterrupted chemotherapy, the three groups were treated as follows: Group A remained on an uninterrupted regimen of 0.05 per cent of isoniazid in the diet, Group B was given isoniazid only once a week (Mondays) for 24 hours, Group C received isoniazid 2 × 24 hours a week (Mondays and Thursdays). Beginning two weeks after infection, 3 randomly selected mice of each of the three groups were sacrificed every Monday and Thursday and bacterial counts were made from their lungs and spleens. Ten randomly selected mice of each of the three groups served as survival controls. Thus, during 7 weeks following the initial chemotherapy period, organ cultures were made a total of fourteen times from 3 mice each of each group, while 10 mice from each group were continued on the respective drug regimens throughout the observation period.

Twelve weeks after infection, the mice of the three control groups still gained weight steadily. As seen in table 1, the mice which were on continuous isoniazid gained less weight than those that received intermittent medication. In order to ascertain that this was not necessarily the expression of a toxic effect of isoniazid, normal mice were kept on 0.05 per cent of isoniazid for the same length of time and their weights recorded. In these animals, isoniazid had no depressing effect on the body weight.

After all 156 experimental mice had been sacrificed, it was decided to use the remaining 30 survival controls for organ cultures, too. Two mice of each group were sacrificed at weekly intervals, thus extending the observation period another five weeks. All nontreated infection controls had died as expected (mean survival: 19 days; range: 16–21 days). The results of the bacterial counts from lungs and spleens of the three chemotherapy groups are summarized in figures 3 and 4. Each point in these figures corresponds to the mean of the logs of the viable counts obtained from 9 plates (triplicate determinations from three organs each time).

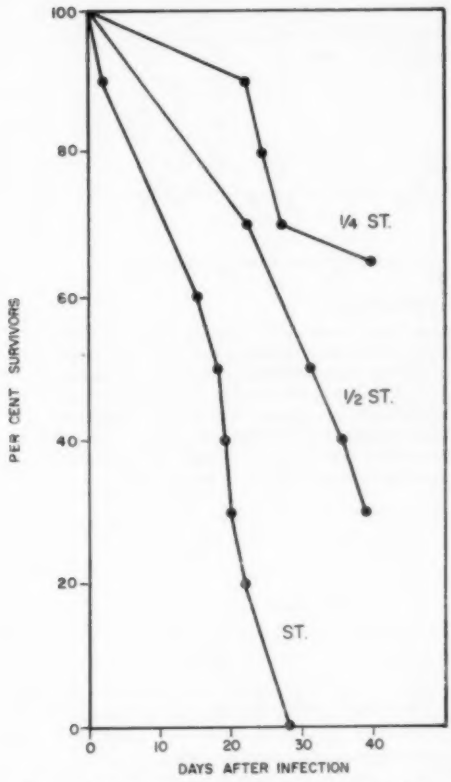

FIG. 2. The survival of mice infected with a standard dose of *M. tuberculosis*, strain Ravenel (ST), one half the standard dose (½ ST), and one fourth of the standard dose (¼ ST).

It is evident from figures 3 and 4 that there were differences in the bacterial numbers found in the organs of the mice of the three groups. In the lungs, Group A, which was on a continuous isoniazid regimen, showed a decline of bacterial numbers extending over the entire observation period of 103 days, i.e., 117 days after infection. This decline was most conspicuous during the first 52 days of treatment and leveled off in the second half of the observation period. In Groups *B* and *C*, which received isoniazid twice or once a week, respectively, the bacterial numbers remained constant throughout the entire observation time.

In the spleens, all three groups showed a steady decline in bacterial numbers throughout the duration of the experiment. Again, the mice that received isoniazid continuously showed the lowest figure (about 30 viable units per organ), but it was only slightly lower than the values found in the spleens of mice receiving isoniazid only once a week for 24 hours, i.e., one seventh of the dose received by Group A.

It must be remembered in this connection that, at the time the mice were put on the three different drug regimens, all animals had been under continuous treatment for 7 days. Therefore, the differences in the one-day values reflect merely the variation between individual animals as they also appear from the data in figures 3 and 4. These inter-animal variations reflect true differences between animals and are not due to sampling or experimental errors. Intra-animal variations were consistently very small.

Resistance to isoniazid and the presence of catalase activity were determined in randomly selected samples from each of the cultures represented in figures 3 and 4. The results of these tests are tabulated in table 2. It may be seen that isoniazid resistance developed in all three groups but that it occurred later and at a slower rate in animals receiving intermittent treatment than in mice which were fed isoniazid continuously. There was good correlation in all instances between the increase in drug resistance and the reduction of catalase activity.

Experiment 3: In *Experiment 3*, an attempt

TABLE 1

AVERAGE BODY WEIGHTS OF MICE UNDER THE INFLUENCE OF ISONIAZID

Group	Drug Regimen	Initial Average Body Weight	Average Body Weight 13 Weeks after Infection
		gm.	*gm.*
A	0.05% Isoniazid continuously	22.6	30.0
B	0.05% Isoniazid twice a week	22.7	35.0
C	0.05% Isoniazid once a week	21.7	34.0
Controls	0.05% Isoniazid continuously	22.8	30.0*
Controls	No isoniazid	23.3	31.8*

* These groups were not infected, but were kept for 13 weeks under comparable conditions.

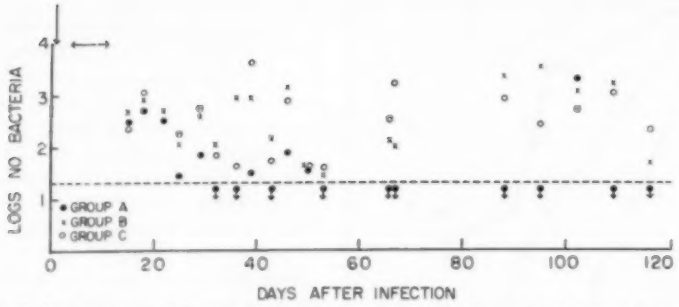

FIG. 3. Ordinate: Logs of the geometric means of the numbers of bacteria cultured from lungs of tuberculous mice. After an initial period of continuous therapy, the mice in Group A received isoniazid uninterruptedly; Group B received isoniazid once a week for 24 hours; Group C received isoniazid twice a week for 24 hours. The horizontal broken line indicates the limit of sensitivity of the method (20 viable bacterial units per organ). The arrowed symbols below this line indicate that the mean counts in these instances were below 20 viable bacterial units. The vertical arrow in the upper left corner indicates the day of infection, the horizontal double arrow, the period at which all mice received continuous isoniazid treatment.

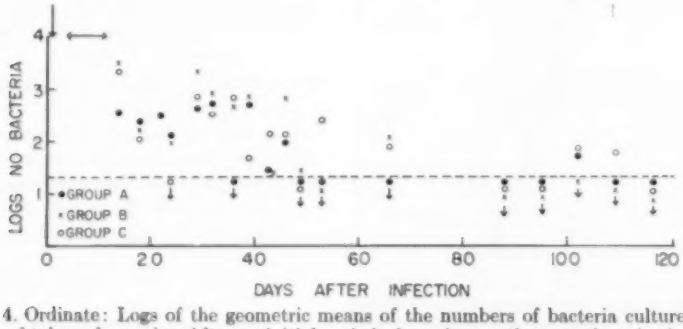

FIG. 4. Ordinate: Logs of the geometric means of the numbers of bacteria cultured from spleens of tuberculous mice. After an initial period of continuous therapy, the mice in Group A received isoniazid uninterruptedly; Group B received isoniazid once a week for 24 hours; Group C received isoniazid twice a week for 24 hours. The horizontal broken line indicates the limit of sensitivity of the method (20 viable bacterial units per organ). The arrowed symbols below this line indicate that the mean counts in these instances were below 20 viable bacterial units. The vertical arrow in the upper left corner indicates the day of infection; the horizontal double arrow, the period at which all mice received continuous isoniazid treatment.

was made to compare continuous and intermittent (24 hours once a week) treatment without a preceding period of continuous treatment for all groups, various intervals of time elapsing between infection and the beginning of treatment. Three days later, 70 mice were infected and subdivided into seven groups of 10 each. Treatment with 0.05 per cent of isoniazid in the diet was started 3, 6, and 10 days after infection. On these days, two groups of 10 mice each were started on a continuous and a once-a-week isoniazid regimen and kept on these schedules throughout the experiment.

The survival of the mice in *Experiment 3* over an observation period of 116 days is shown in table 3. The average weights of the survivors are seen in figure 5. It may be noted that there was no significant difference between the effects of continuous treatment in the two groups of mice when medication began 3 and 6 days after infection. This seems remarkable in view of the fact that, in this experiment, the median survival time of the untreated controls was only 18 days. When treatment was begun only 10 days after infection, however, the intermittent treatment was clearly inferior to the continuous regimen, and a number of mice

TABLE 2

ISONIAZID RESISTANCE OF TUBERCLE BACILLI CULTURED FROM THE LUNGS OF MICE IN EXPERIMENT 2

Group	Days after Infection	Isoniazid Concentration in the Medium (γ/ml.)						
		0.1	0.5	1.0	5.0	10.0	50.0	100.0
A	19	18*	12	5	1	0	0	0
B	19	++	7	6	1	0	0	0
C	19	+	4	6	2	0	0	0
A	39	+++	+++	++	+	19	3	0
B	39	++	+	11	3	1	0	0
C	39	++	++	+	7	0	0	0
A	65	+++	+++	+++	++	++	18	2
B	65	+++	+	10	11	2	0	0
C	65	+++	+	+	+	15	1	0
A	121	+++	+++	+++	+++	+++	++	+
B	121	+++	+++	+	19	4	0	0
C	121	+++	+++	+	++	14	4	0

* The figures or symbols indicate the average number of colonies grown on these media. The colonies were counted up to 20 per slant, and the growth intensity estimated as + to +++ when the number of colonies exceeded 20.

could not be saved with the once-a-week regimen of isoniazid. Those who survived recovered and seemed to do about as well as the other groups (*see* weight curves, figure 5).

These observations suggested that, with very severe infections, an initial period of intensive continuous treatment was necessary to suppress the number of viable bacteria to a sufficiently low level when it could then be controlled with intermittent therapy. In the following experiment this problem was studied further, using also smaller doses of isoniazid.

Experiment 4: One hundred and forty mice were infected with the standard dose of *M. tuberculosis*, strain Ravenel, and randomly distributed in seven cages of 20 mice each 6 days later. Six groups were then given 0.05 per cent of isoniazid in the diet for 7 days, and from then on were treated as described in table 4.

With the highest dose of isoniazid (0.1 per cent), there appeared to be no difference between continuous and intermittent regimens as judged from survival of these animals. Thus, an initial one-week period of intensive treatment reduced the number of bacteria sufficiently so that an intermittent drug regimen seemed to be able to control the disease from there on. However, when the concentration of isoniazid in the food was lowered to 0.05 or 0.01 per cent, the intermittent regimens were inferior to the continuous medication.

Drug resistance was not tested in these mice, but it is likely that the late survivors had tubercle bacilli highly resistant to isoniazid which would make the observed differences in the response to higher and lower drug concentrations understandable. The experiment was discontinued 160 days after infection.

Experiment 5: Since it appeared from the above experiments that adequate doses of isoniazid given intermittently were able to control infections as long as the disease was not too severe, an experiment was set up in which 250 mice were infected intravenously with only about 3,000 viable units of tubercle bacilli, as determined by plate counts of the inoculum. Four different drug regimens were compared in this experiment:

Group A: Controls, no medication

Group B: Isoniazid, 0.1 per cent in the diet, 24 hours per week, beginning 6 days after infection

Group C: Isoniazid, 0.01 per cent in the diet, 24 hours per week, beginning 6 days after infection

Group D: Isoniazid fed by stomach tube, 0.2 mg. in 0.2 ml. of water six days a week, beginning 7 days after infection

Group E: Isoniazid fed by stomach tube, 2 mg. in 0.2 ml. of water once a week, beginning 3 weeks after infection.

Three mice of every group were randomly selected and sacrificed at weekly intervals and the

TABLE 3

SURVIVAL OF MICE ON CONTINUOUS AND INTERMITTENT (ONCE-A-WEEK) ISONIAZID TREATMENT STARTED AT VARIOUS TIME INTERVALS AFTER INFECTION

Kind of Treatment*	Individual Survival (Days after Infection)	Number of Survivors 116 Days after Infection
No treatment (infection controls)	14, 18, 18, 18, 18, 19, 23, 24, 28, 30	0
Isoniazid continuously, beginning 3 days after infection	17, 52	8
Isoniazid intermittently, beginning 3 days after infection		10
Isoniazid continuously, beginning 6 days after infection		10
Isoniazid intermittently, beginning 6 days after infection	34	9
Isoniazid continuously, beginning 10 days after infection	13	9
Isoniazid intermittently, beginning 10 days after infection	12, 12, 12, 13, 19, 40	4

* Treatment: Isoniazid, 0.05 per cent in diet continuously, or once a week for 24 hours.

number of viable bacteria determined in the lungs. Whenever the plates were counted, all colonies were checked for the presence of peroxidase (4). The experiment lasted 18 weeks.

First, it should be mentioned that no isoniazid resistance developed in any of the mice throughout the duration of this experiment. All colonies remained peroxidase positive.

Of the untreated control mice, 2 died during the observation period. Of a survival control group infected at the same time, 50 per cent of the animals had died 135 days after infection (range 109 to 193 days).

None of the isoniazid-treated mice died before they were to be sacrificed, but their organs yielded quite different numbers of viable bacteria (figure 6). In Group B, which received isoniazid as a 0.1 per cent addition to the diet during 24 hours a week, the bacterial counts dropped below the theoretic limit of 20 organisms per lung which was detectable by the culture method employed. This drop occurred after the first day of medication and, with two exceptions, the lung cultures from these mice remained sterile throughout. On the contrary, isoniazid had only a limited effect on bacterial counts when given in the diet in a 0.01 per cent concentration, or when it was fed by stomach tube at dosages of 0.2 mg. per day, six days a week, or 2 mg. per day once a week.

It is estimated that a mouse takes in 3 to 4 gm. of food per day. The mice in Group B would thus have eaten about 3 to 4 mg. of isoniazid over a 24-hour period, once a week, and it was somewhat surprising that the bacterial counts in Group E (2-mg. dose) should have remained as high as they were. An attempt was therefore made to determine drug concentrations in the blood of mice after feeding continuously and receiving the drug by stomach tube.

Experiment 6: Mice were fed diets containing 0.1, 0.05, and 0.01 per cent of isoniazid for 24 hours. They were then put on unmedicated food and bled at intervals. The serum was filtered through millipore filters and added to oleic-acid-albumin medium in various concentrations, ranging from 0.1 ml. per ml. to 0.01 ml. per ml. The tubes were inoculated with *M. tuberculosis*, strain Ravenel, and checked for bacterial growth after one and two weeks. The susceptibility of the bacteria to isoniazid was determined in control tubes containing various concentrations of isoniazid with and without normal mouse serum.

The results are summarized in table 5. Isoniazid alone, or in the presence of 10 per cent of normal mouse serum, inhibited the growth of tubercle bacilli completely in a concentration of 0.05–0.1 γ per ml. The results reported in table 5 indicate, therefore, that, while the mice were fed a diet containing 0.1 per cent of isoniazid, their serum concentrations of isoniazid were in the order of 10 γ per ml., but that only one hour after discontinuing the medication, the drug concentrations had already fallen below 1 γ per ml. With 0.05 per cent of isoniazid in the diet, serum concentrations of about 4 γ per ml. were present as long as the mice were feeding whereas, with 0.01 per cent of isoniazid in the diet, serum concentrations above 1 γ per ml. were never obtained.

In a similar experiment, isoniazid serum con-

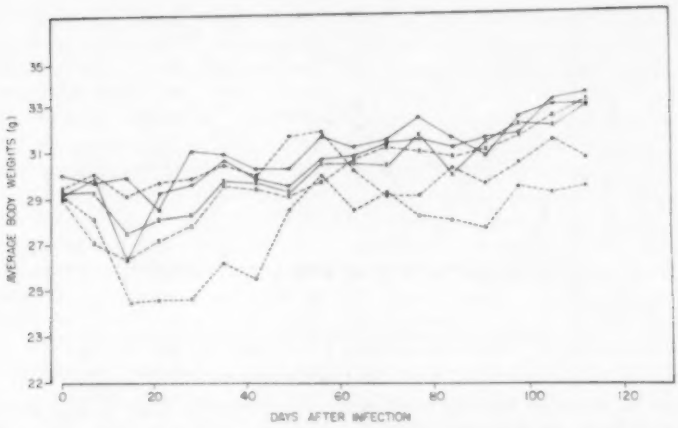

FIG. 5. Average weights of mice treated with various regimens of isoniazid, 0.05% in the diet—

·——· Isoniazid continuously, beginning 3 days after infection.
·-----· Isoniazid 24 hours a week, beginning 3 days after infection.
×——× Isoniazid continuously, beginning 6 days after infection.
×-----× Isoniazid 24 hours a week, beginning 6 days after infection.
○——○ Isoniazid continuously, beginning 10 days after infection.
○-----○ Isoniazid 24 hours a week, beginning 10 days after infection.

TABLE 4

EFFECTS OF INTERMITTENT AND CONTINUOUS ISONIAZID TREATMENT AT DIFFERENT DOSAGE LEVELS

Groups and Drug Regimen*	Individual Survival Times	Number of Survivors 160 Days after Infection	Average Weight of Survivors 160 Days after Infection
	days		*gm.*
Infection controls, none	13, 14, 15, 17, 17, 17, 17, 17, 18, 18, 18, 19, 19, 20, 20, 20, 21, 21, 21, 24	0	—
Isoniazid 0.1% continuously	19	19	32.1
Isoniazid 0.1% 1 day a week	40	19	32.9
Isoniazid 0.05% continuously	30, 34, 37, 68	16	33.2
Isoniazid 0.05% 1 day a week	21, 102, 102, 108, 112, 119, 121, 123, 123, 126, 130, 136	7	35.8
Isoniazid 0.01% continuously	31, 47, 66, 132	16	33.0
Isoniazid 0.01% 1 day a week	14, 23, 29, 108, 121, 132, 133, 147, 149, 150, 155	9	31.5

* All mice (except the infection controls) were treated with isoniazid, 0.05 per cent continuously for one week, beginning 6 days after infection. Following this initial treatment, the various regimens mentioned below were initiated.

centrations were determined at various intervals after feeding 2 mg. of isoniazid by stomach tube. The results of the biologic testing for isoniazid in these serum specimens are tabulated in table 6. Up to two hours after feeding, the concentrations of biologically active isoniazid were still in the order of about 20 γ per ml., i.e., higher concentrations were reached by feeding the drug in one dose. These values are slightly higher than those reported by Canetti and associates (7), but the small differences can easily be attributed to variations in the tech-

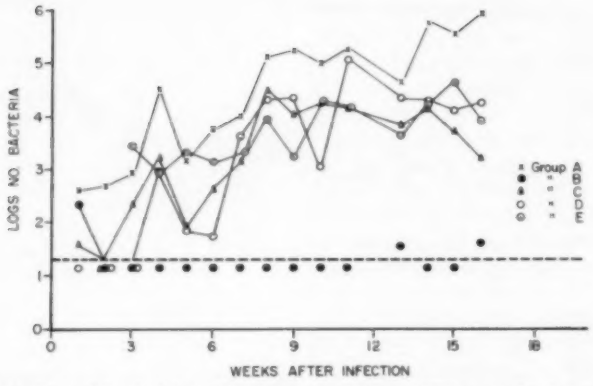

FIG. 6. Mean logs of bacterial numbers recovered from the lungs of mice receiving the following different regimens of isoniazid.

Group A: Infection controls, no isoniazid.

Group B: Isoniazid 0.1 per cent in diet, 24 hours per week, beginning 6 days after infection.

Group C: Isoniazid, 0.01 per cent in diet, 24 hours per week, beginning 6 days after infection.

Group D: Isoniazid, 0.2 mg., fed by stomach tube 6 days a week, beginning 7 days after infection.

Group E: Isoniazid, 2.0 mg., fed by stomach tube once a week, beginning 21 days after infection. The horizontal broken line indicates the limit of sensitivity of the method (20 viable units per log). All symbols below the broken line indicate sterile cultures, i.e., organs containing fewer than 20 viable bacterial units.

TABLE 5

IN VITRO GROWTH INHIBITION OF *M. TUBERCULOSIS*, STRAIN RAVENEL, IN THE PRESENCE OF SERUM SPECIMENS OBTAINED FROM MICE ON ISONIAZID-CONTAINING DIETS

Per Cent of Isoniazid in Diet	Hours after Cessation of Medication at Which Serum Was Obtained	Concentration of Serum in Medium	Growth Intensity
		per cent	
0.1	0	10	0
0.1	0	5	0
0.1	0	2.5	0
0.1	0	1.25	0
0.1	0	0.6	±
0.1	0	0.3	++
0.05	0	10	0
0.05	0	5	0
0.05	0	2.5	0
0.05	0	1.25	++
0.01	0	10	++
0.1	1	10	++
0.05	1	10	++
0.01	1	10	++

TABLE 6

IN VITRO GROWTH INHIBITION OF *M. TUBERCULOSIS*, STRAIN RAVENEL, IN THE PRESENCE OF SERUM SPECIMENS FROM MICE BLED TWO AND FOUR HOURS AFTER THEY WERE FED 2 MG. OF ISONIAZID*

Final Serum Concentration in Medium	Bacterial Growth with Serum from 2 Hour Bleedings	Bacterial Growth with Serum from 4 Hour Bleedings
per cent		
10	0	+
5	0	+++
2.5	0	+++
1.2	0	+++
0.6	0	+++
0.3	++	+++
0.0	+++	+++

* In the controls to this experiment, bacterial growth was completely inhibited by 0.06 γ of isoniazid per ml.

nique as well as the strain of mice used. However, four hours after feeding, all detectable isoniazid had disappeared from the blood.

DISCUSSION

Intermittent chemotherapy has been studied in mice before (8, 9). However, these early

studies do not seem to have been repeated, nor was it possible to find records of attempts to apply these findings to clinical treatment of tuberculosis except for a brief report by Rothstein and Johnson (10) who found that one weekly dose of 2 gm. of streptomycin was about as effective as more frequent injections of the antimicrobial. In a long series of papers, Bartmann and co-workers (11) compared the effects of continuous and intermittent doses of isoniazid in experimental tuberculosis of guinea pigs. "Continuous" in the terminology of those writers signified 6 weekly injections of 5 or 10 mg. of isoniazid per kg. of body weight, whereas "intermittent" treatment consisted of 2 such doses a week. As a whole, the results of the continuous treatment were somewhat better than with intermittent therapy, but the differences were slight and good results were obtained with the less frequent injections. Palmer and associates (12), who studied the effects of various isoniazid regimens in large groups of heavily infected guinea pigs, came to the conclusion that "a given amount of drug appears to be more effective in large, less frequent doses than in small, more frequent doses." Thus, in their experiments, a single weekly dose of 20 mg. of isoniazid per kg. was more effective than a daily dose of 3 mg. per kg.

The present experiments were undertaken with a practical application in mind. It is submitted that if isoniazid could be given to ambulatory patients as infrequently as once a week, this would be of the greatest value for mass treatment schemes when controls to assure a regular drug intake would be difficult to set up. The question which these experiments tried to answer was therefore not whether intermittent treatment would yield *better* results than any of the clinically proved regimens, but rather whether *approximately as good* results could be obtained by intermittent medication.

To a large extent, the present results seem to answer the above question in the affirmative. The once-a-week regimens compared favorably with the continuous treatment schemes, as long as the dosages were sufficiently high. With lower doses, a limit was reached at which the intermittent treatment was clearly inferior to continuous medication; but this disadvantage could, to some extent, be corrected by higher single doses. The values of the viable counts obtained in *Experiments 2* and *5* correspond well with the data published by McCune and Tompsett (13) and by Canetti and associates (7).

Dosage levels are difficult to compare in mice and men, and conclusions must be drawn with care. Single doses of 2 mg. per mouse, corresponding to 80 to 100 mg. per kg. of body weight, are apparently well tolerated in mice, but adequate therapeutic results are also obtained with smaller doses (7). If, as is usually done, the drug is added to the diet, 0.01 per cent is about the lowest dosage at which severe acute infections are controlled. While the progress of tuberculosis is checked at this level, the infection is not eradicated (13). Since an adult mouse consumes about 4 gm. of food per day, the daily drug intake here approximates 0.4 mg., or 20 mg. per kg. Mice feed irregularly and frequently during most of 24 hours. The drug intake at each feeding is therefore low; but blood concentrations, although low ones, are maintained throughout. On the contrary, feeding isoniazid by stomach tube results in higher blood concentrations of shorter duration.

Concentrations of active isoniazid seem to persist longer in man than in mice. With doses of 5 mg. per kg., Bartmann and Massmann (14) found drug concentrations of above 1 γ per ml. to persist an average of 6 hours. At 2 hours, the average value was 4 γ per ml. Similar results were reported by Mount and co-workers (15). In contrast, with a comparable dosage in mice, Canetti and associates (7) found the concentrations of active drug 2 hours after drug intake to be ten to twenty times lower (0.25–0.50 γ per ml.). The present findings (tables 5 and 6) are similar to those of the French workers. Thus, elimination of active isoniazid from the blood is much faster in mice than in human patients and higher doses must be chosen to obtain results which might be comparable to the human situation. The rapid disappearance of active isoniazid from the serum of mice is undoubtedly also the reason why, in *Experiment 5*, the group which ingested an estimated 3 to 4 mg. of isoniazid over a period of 24 hours, once a week, had so much lower bacterial counts in the lungs than the group that received about half this dose, i.e., 2 mg., once a week, in one single feeding.

In mice infected with large doses of tubercle bacilli and treated with isoniazid, drug resist-

ance occurred in all instances, but the levels of microbial resistance reached during the observation periods were lower in the intermittently treated animals than in the mice that received isoniazid continuously. If this observation can be confirmed, it would be of interest, especially when intermittent treatment schemes with isoniazid alone are considered under field conditions. With small infective doses (*Experiment 5*), drug resistance failed to develop throughout the observation period.

From a theoretic point of view, it is not too surprising that isoniazid given only once a week should be effective as long as sufficiently high doses are chosen. The bactericidal effect of the drug is much more pronounced when it acts on bacteria which are in an active state of multiplication, whereas resting bacteria are much less affected by the drug (16–19). It may be expected then that the impact of isoniazid *in vivo* is greatest when the drug acts on rapidly multiplying bacteria. Indeed, a great many studies have shown that under the influence of isoniazid (as well as of other agents), the number of viable bacteria which are recovered from animal organs drops sharply at the beginning of treatment, but becomes less and less affected as time goes on. With isoniazid given intermittently, there is sufficient time in the drug-free intervals for the bacteria to resume active multiplication and thus become more susceptible again when the next drug impact occurs. Relative to the long generation time of tubercle bacilli (16 to 24 hours), a regimen in which effective drug concentrations in blood and tissues are reached for a few hours once a week might be comparable to that in infections with gram-positive cocci with a generation time of 20 to 30 minutes when penicillin is given once or twice a day.

Semiquantitative experiments of Armstrong (20) have shown that a daily contact of tubercle bacilli *in vitro* with as little as 1 γ per ml. of isoniazid for only 2 hours had a complete bacteriostatic effect. Longer contacts with higher drug concentrations were needed, however, for bactericidal effects to become manifest. Although it is difficult to draw conclusions from observations *in vitro*, such as these, on conditions as they prevail *in vivo*, there is no doubt that marked bactericidal effects did occur in the present experiments *in vivo*.

As mentioned before, these experiments were undertaken with a practical goal in mind: To study under laboratory conditions treatment schemes which might be applicable in patients, especially under difficult field conditions. There, the public health officer is often faced with a choice between a well established method which is difficult to apply for want of the necessary installations and means, and trying a new procedure which has a chance of giving acceptable results. It is suggested that once-a-week treatment with high doses of isoniazid might be a reasonable substitute for hospitalization, especially if it is preceded in more advanced cases by a short (perhaps 3-week) intensive period of classical chemotherapy in a hospital.

In order to get some information as to the dosage which might be used, some preliminary tests were done in a few normal human volunteers. It was found that 30 mg. of isoniazid given once a week in one single oral dose were well tolerated over a period of 2 months. Preliminary trials in patients are now under way which seem to confirm these findings. Obviously, the final answer as to the adequacy of such treatment can only come from carefully controlled clinical studies.

Summary

The therapeutic effects of isoniazid given continuously were compared in tuberculous mice with the results obtained with regimens in which the drug was given intermittently. The action of the drug was judged by its effects on body weight, survival, and numbers of viable tubercle bacilli cultured at various intervals from lungs and spleens. It was found that in severe, acute, as well as in chronic, murine tuberculosis, the therapeutic results obtained with intermittent drug regimens compared favorably with the effects of continuous treatment. Since good results were observed with one single weekly dose of isoniazid, it is suggested that similar treatment schemes be tried in ambulatory human patients. The possibility of being able to supervise the once-a-week drug intake might represent a considerable advantage over conventional regimens. Bacterial resistance to isoniazid appeared to develop at a slower rate in mice on intermittent treatment schemes than was the case in animals that received isoniazid continuously. In order to be effective when given once a week, the single doses of isoniazid must be relatively large.

Sumario

La Isoniacidoterapia Intermitente en los Ratones

Se compararon los efectos terapéuticos de la isoniacida administrada continuamente a ratones tuberculosos con los resultados obtenidos con regímenes en los que se administró la droga intermitentemente. La acción del medicamento fué juzgada por sus efectos sobre el peso vivo, la sobrevivencia, y el número de bacilos tuberculosos viables cultivados a varios plazos de los pulmones y los bazos. Se observó que, en la tuberculosis murina aguda grave, así como en la crónica, los resultados terapéuticos obtenidos con los regímenes intermitentes se comparaban favorablemente con los alcanzados con el tratamiento continuo. Como se observaron buenos resultados con una sola dosis semanal de isoniacida, se propone que se pruebe un tratamiento semejante en enfermos humanos ambulantes. La posibilidad de poderse vigilar la toma semanal de la droga podría constituir una ventaja considerable sobre los regímenes convencionales. La resistencia bacteriana a la isoniacida pareció formarse con mayor lentitud en los ratones sometidos a planes terapéuticos intermitentes que en los animales que recibieron isoniacida continuamente. A fin de que sea eficaz cuando se administra una vez semanal, la dosis única de isoniacida tiene que ser relativemente grande.

Resume

Thérapeutique intermittente par l'isoniazide chez des souris

On compara les effets thérapeutiques de l'isoniazide donné d'une façon continue chez des souris tuberculeuses, aux résultats obtenus avec un système où le médicament fut donné par intermittence. On jugea l'action du médicament d'après son effet sur le poids corporel, la survie, et le nombre de bacilles tuberculeux viables cultivés à des intervalles variés à partir des poumons et des rates. On trouva que, dans la tuberculose murine sevère, aiguë, aussi bien que dans la tuberculose chronique, les résultats thérapeutiques obtenus avec le système intermittent de médication peuvent être comparés avec succés aux effets du traitement continu. Comme on observa de bons résultats avec une seule dose hebdomadaire d'isoniazide, on propose que des shémas de traitement semblables soient essayés chez des malades humains ambulatoires. La possibilité de pouvoir contrôler la prise hebdomadaire du médicament représente un avantage considérable sur les systèmes habituels. La résistance bactérienne à l'isoniazide semble se développer à un rythme plus lent, chez les souris, avec le mode de traitement intermittent que dans les cas où les animaux reçurent de l'isoniazide de façon continue. Afin d'être efficace, lorsqu'on les donne une fois par semaine, les doses uniques doivent être relativement fortes.

Acknowledgment

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A BACTERIOLOGIC AND HISTOPATHOLOGIC STUDY OF SKIN GRANULOMA DUE TO MYCOBACTERIUM BALNEI[1]

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INTRODUCTION

Tuberculoid skin lesions on the bridge of the nose following injuries sustained while swimming in a public pool were first reported from Stockholm by Hellerström (1) in 1939. In 1951, the same writer (2) was able to report on a total of six cases. Eight cases of similar lesions either on the chin, finger, or nose following swimming in a public pool in Vancouver were reported by Cleveland (3). Nine other cases of lesions on the knee, finger, or ankle contracted in a swimming pool in San Francisco were reported by Rees and Bennett (4) in 1953. The latter investigators, incidentally, refer to ten or twelve other cases of the disease acquired in a swimming pool in Seattle. The cause of the disease was not elucidated in any of the above cases.

An outbreak of tuberculoid lesions on the elbow following swimming in a public pool and affecting some eighty people was observed from 1949 to 1950 in the Swedish town of Örebro. Linell and Nordén (5) identified a Mycobacterium as the causative agent and gave it the name *Mycobacterium balnei*. The same bacterium was found by Zettergren and associates (6) during an outbreak involving some sixty cases in 1951 and 1952 in another Swedish town. During the summer of 1959, a similar outbreak involving about three hundred cases was observed in Colorado. The source of the infection was an open-air, hot springs–mineral water public swimming pool in Glenwood Springs which was polluted with *M. balnei*. The history, epidemiology, and clinical aspects of this outbreak were reported by Mollohan and Romer (7). The bacteriologic and histologic investigations concerned with the identification of the causative agent are reported in the present study.

MATERIAL AND METHODS

The skin lesions made their appearance two to three weeks after infection. In most cases they were localized on the elbow and, less often, on the knee, foot, finger, or toe. The lesions consisted of papules or nodules which slowly increased in size, sometimes ulcerated, or became covered by a scab (figure 1). Healing in the majority of cases occurred spontaneously after several months, often leaving a scar (figure 2). Biopsy specimens of skin lesions submitted by local physicians were used for bacteriologic and histologic study.[2] For histologic study, a portion of the specimen was fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin and by Kinyoun's staining procedure for acid-fast bacteria. Another portion of the specimen, to be used for bacteriologic study, was transferred to a sterile mortar, minced with scissors, and ground with sand under stepwise addition of about 2 ml. of a 0.2 per cent solution of bovine albumin fraction V.[3] A smear was made to detect the presence of acid-fast or other microorganisms. In patients with evidence of secondary infection, a part of the tissue suspension was submitted to a decontamination procedure by mixing with an equal volume of N sodium hydroxide, incubating for ten minutes, and then neutralizing with N hydrochloric acid in the presence of bromthymol blue as an indicator. The suspension was inoculated on oleic acid–albumin agar medium, 7H-10 (8), by pipetting 0.1 ml. in the center of the plate and spreading by means of a sterile L-shaped Pasteur pipette while the plate was revolving on a turntable. The plates were enclosed in polyethylene bags and incubated at 30 to 33°C. for a period of two weeks. At least five plates were used for each specimen. Samples of water from the swimming pool which was suspected of being the source of the epidemic were handled in a similar manner.

The properties of the strains isolated in this investigation were compared with those of the following strains:

(*1*) Four strains of *M. balnei* isolated in the Swedish epidemics (ATCC 11564, 11565, 11566, and 11567).

(*2*) Four strains of mycobacteria[4] isolated in Hawaii from patients with granulomatous skin lesions on the knee, finger, or hand.

[1] From the Department of Experimental Microbiology, Division of Research and Laboratories, National Jewish Hospital at Denver; and the Department of Microbiology, University of Colorado School of Medicine, Denver, Colorado.

[2] The writers wish to acknowledge the collaboration of the following physicians, who supplied the biopsy specimens: Drs. Henry M. Lewis, Denver; Robert C. Lewis, B. E. Nutting, and G. W. Horst, Glenwood Springs; John P. Hyland, Grand Junction; and Harry O. Hendrick, Carbondale.

[3] Pentex, Incorporated, Kankakee, Illinois.

[4] The writers' thanks are due Dr. Hastings Walker, Medical Director, and W. F. Shinn, Chief Laboratory Technician, Leahi Hospital, Honolulu, Hawaii, for sending these strains and clinical data.

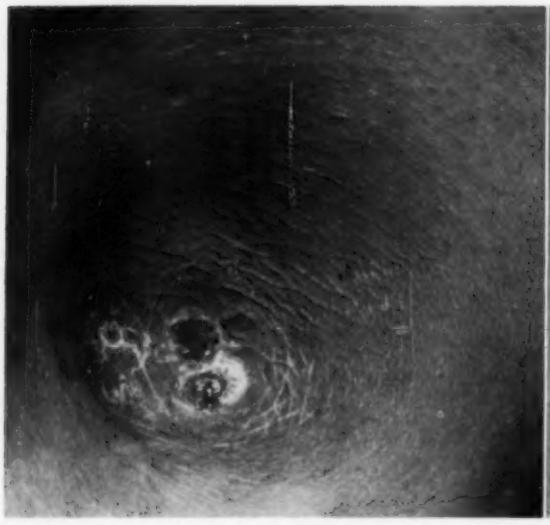

FIG. 1 (Left). Skin lesion on the elbow with scab formation, six weeks' duration.
FIG. 2 (Right). Scar formation six months after infection.

These infections had been contracted on the beaches of various Hawaiian islands.

(3) Two strains of mycobacteria[a] isolated from diseased fish in aquariums, namely *M. marinum* (ATCC 927) isolated by F. D. Aronson (9) in 1926 from salt-water fish in Philadelphia, and *M. platypoecilus* isolated by Baker and Hagan (10) in 1942 from tropical fish.

(4) Numerous strains of *M. kansasii* (11) isolated from patients with pulmonary disease resembling tuberculosis. Strains of this type were chosen for comparison because they resemble *M. balnei* in their cellular and colonial morphology, including color change on exposure to light, but differ by a higher temperature optimum for growth and their pathogenic effects in the lungs of mice on air-borne infection.

RESULTS

Bacteriology

Skin lesions from 24 patients were received for investigation during a period in excess of eighteen months. The specimens came from 19 patients with lesions on the elbow; 2 patients with lesions on the knee; and 3 patients with lesions on the toe, dorsum of the tibia, or foot. The lesions in the latter patients were secondary to a primary lesion on the toe. The results of the culture are summarized in table 1. *Mycobacterium balnei* was isolated from 17 patients; 13 had lesions on the elbow and the others had lesions on the knee, toe, dorsum of the foot, or tibia. In 2 patients the elbow lesions were complicated by bursitis. Some of the fresher lesions yielded numerous colonies on each plate. Lesions three-months old or older generally yielded only a few colonies. It is of interest that the twelve- and eighteen-month-old lesions were still positive for *M. balnei* on culture. The failure to cul-

TABLE 1

RESULTS OF THE CULTURE OF MYCOBACTERIUM BALNEI FROM BIOPSY SPECIMENS ACCORDING TO THE AGE OF THE LESIONS

Approximate Age of Lesion	Total Number of Specimens Examined	Culture Result	
		Positive	Negative
months			
1–4	13	9	4
6–8	9	6	3
12–18	2	2	0
Total	24	17	7

[a] The writers are thankful to Dr. Charles Shepard, U. S. Public Health Service, Communicable Disease Center, Atlanta, Georgia, for having called attention to the similarity of these strains to *M. balnei*.

ture the organisms from some of the specimens may have been due to the badly desiccated state in which they arrived at the laboratory.

In all except one instance, cultivation of *M. balnei* was achieved by direct inoculation of the ground specimen without preceding decontamination. Four specimens were inoculated without and with preceding decontamination; two were positive on direct culture and negative when treated by the decontamination procedure. One specimen was positive by both methods, but the decontaminated part yielded one-fifth as many colonies as the untreated part. One lesion with secondary infection gave a mixture of staphylococci and mycobacteria on direct culture and a pure culture of *M. balnei* on decontamination. The colonies of *M. balnei* on the directly inoculated plates could be distinguished from those of the staphylococci by their characteristic morphology. These observations suggest that the chances of isolating *M. balnei* from biopsy specimens are best when the material is inoculated directly, and that this method can be recommended even if secondary infection is present. In the latter case, a parallel culture may be made from the material after decontamination.

Samples of water from the swimming pool were inoculated directly without decontamination and in ten- and one hundred fold dilutions. At the time of the height of the epidemic they yielded as many as a thousand colonies of *M. balnei* per ml. of water. Later tests which were made several days after the walls of the pool had been washed with a concentrated chlorine solution gave no growth of *M. balnei* from the pool water, but the bacteria could still be cultivated from swabs made from fissures in the walls of the pool.

To determine the optimal growth temperature of *M. balnei* on primary culture, two specimens were incubated simultaneously at 30° and 37°C. Incubation at 37°C. was done in a walk-in incubator furnished with a tightly closed cabinet into which air containing 5 per cent carbon dioxide was introduced once daily. This procedure was used because it markedly improves the growth of tubercle bacilli and *M. kansasii* on oleic-acid-albumin agar in Petri dishes (12). Both specimens gave growth at 30° and 37°C., but the number of colonies at 30°C. was three times greater than at 37°C. No growth of *M. balnei* was obtained under incubation at 37°C. in atmospheric air.

The colonies of *M. balnei* in cultures at 30°C. became visible between the eighth and fourteenth day of incubation. They were slightly wrinkled and shiny, sometimes perfectly smooth and hemispherical and, in a single case, rough and dry. With increase in size their color became whitish and they lost their transparency. Exposure to daylight for a few hours caused a progressive change of color to yellow-orange within a day or two. This photochromogenic effect distinguishes colonies of *M. balnei* from those of "scotochromogenic" mycobacteria, which are yellow before exposure to light. This latter type of mycobacteria is occasionally present in swimming pool waters, but does not cause skin infections.

The pathogenic properties of the strains of *M. balnei* were studied in guinea pigs and CF_1 mice. No pathologic effects were seen in guinea pigs on subcutaneous injection or air-borne infection. Intravenous inoculation of four white mice with 2.5 million bacteria produced a lesion in the tail at the site of the injection in all of them, but only one internal lesion occurred, in a mouse which had a testicular abscess containing numerous acid-fast bacteria. Intraperitoneal injection of an undiluted or tenfold diluted culture from Tween®-albumin liquid medium into male mice caused pronounced swelling of the scrotal sac, which was followed in some animals by necrosis of the scrotal skin. Intradermal injection of a million bacteria into the footpads of mice, according to the method of Fenner (13), caused swelling of the footpad and redness within a few days, progressing slowly to a yellowish discoloration and, sometimes, ulceration. A one hundred fold smaller concentration of bacteria caused a similar lesion which developed more slowly. Exposure of mice to air-borne infection in the chamber devised by Middlebrook (14) did not produce lesions except in rare cases in which a few tubercles were seen in the lungs. Two of the Hawaiian strains, however, seemed to have a greater tendency to produce lung lesions. These results were similar to those obtained with the Swedish strains. They showed that the pathogenic effects of *M. balnei* in most cases are limited to the skin and to those tissues which have a lower temperature than the internal organs.

In order to study the antigenic specificity of *M. balnei* and *M. kansasii*, rabbits were immunized by repeated intravenous injections of heat-

killed bacterial suspensions for a period of several weeks. The antiserum was studied for its agglutinating effect on suspensions of *M. balnei* and other acid-fast bacteria in 0.5 phenol containing phosphate-buffered saline. The results were read after incubation overnight at 37°C. The agglutinating effect could be judged best by the clearing of the supernatant in the presence of the antiserum, as compared to the saline control which retained its cloudiness or opalescence. It was found that the suspensions of all strains classified as *M. balnei* were agglutinated by the *M. balnei* antiserum to a high titer, whereas the suspensions of *M. kansasii* or other mycobacterial types were agglutinated only to a much lower titer. Antiserum prepared with a strain of *M. kansasii*, on the contrary, agglutinated all strains of *M. kansasii* which could be obtained in stable suspension, but none of the strains of *M. balnei* or other mycobacteria. This result indicated that *M. balnei* and *M. kansasii* are antigenically distinct. It also showed that the strains from Sweden, Hawaii, and Colorado, and *M. platypoecilus* belong to the same type.[6] The specificity of the tuberculin reactions in guinea pigs sensitized with strains of *M. balnei* from Sweden and Colorado, as well as a strain of *M. kansasii*, was also studied. Filtrates from two- to three-month-old cultures in Wong medium were used as tuberculins. These filtrates had been standardized on tuberculous patients by determining the dilution which on intradermal injection gave a tuberculin reaction of the same strength as that produced by a 1:1,000 dilution of OT. These dilutions were used in the guinea pigs. The results of these tests showed that the animals sensitized with the strains of *M. balnei* gave the strongest reactions with the two filtrates from the *M. balnei* strains and weaker reactions with the filtrate from *M. kansasii* or OT, whereas the animals sensitized with *M. kansasii* reacted most intensely with the homologous filtrates. These results thus confirmed the identity of the Swedish and Colorado strains of *M. balnei* and their antigenic difference from *M. kansasii*. The latter result was also reported recently in studies by Magnusson and associates (15, 16).

A special study was made of the usefulness of the optimal growth temperature as a criterion for the differentiation of *M. balnei* and *M. kansasii*. Linell and Nordén (5) had shown that the ability of strains of *M. balnei* to grow at 37°C. depended on the composition of the culture medium; only glycerol agar, but not Löwenstein-Jensen medium, supported growth at this temperature. Using oleic-acid-albumin agar medium, the writers found that the growth of *M. balnei* at 37°C. in a carbon dioxide–enriched atmosphere was variable and often nearly as good as at 30°C. This medium, therefore, did not permit a clear-cut differentiation between strains of *M. balnei* and *M. kansasii*. In order to determine whether under other, perhaps less favorable, growth conditions the temperature of incubation might be a more critical factor for growth and thus permit differentiation between these two types, tubes of liquid Tween-albumin medium were inoculated with small amounts of *M. balnei* or *M. kansasii*, and growth at 30° and 37°C. in ordinary incubators was compared. It was found that all twenty-two strains of *M. balnei* grew very well at 30°C. but not at all or very poorly at 37°C., whereas an equal number of strains of *M. kansasii* grew as well or even better at 37° than at 30°C. This test, therefore, can be used for differentiation between the two mycobacteria.

Studies by Linell and Nordén of the susceptibility of *M. balnei* to various drugs showed that the bacteria were resistant to isoniazid, para-aminosalicylic acid, and thiosemicarbazone, and that they were partially susceptible to 4 γ per ml. and fully susceptible to 8 γ per ml. of streptomycin. Similar studies carried out by incorporating the drugs in oleic-acid-albumin agar medium and using as inoculum a thousand-fold dilution of cultures of several strains of *M. balnei* in liquid Tween-albumin medium yielded the results summarized in table 2. Although some of these drugs have a marked inhibiting effect *in vitro*, their systemic use seems to be excluded in view of the self-healing nature of the disease. Topical application also would seem to be of no value because of the inability of the drug to penetrate through the skin.

Histopathology

A histologic examination was made of 15 of the 24 specimens received for culture studies. The age of the skin lesions at the time of biopsy ranged from one to eighteen months, the majority being six months or less.

[6] Suspensions of *M. marinum* agglutinated spontaneously and therefore could not be studied by this method.

The cutaneous lesions were usually limited to the corium, and seldom extended beyond the level of the sweat glands. In two instances, however, the infection extended into the bursa above the olecranon. The lesions commonly had a slight-to-moderate degree of hyperplasia of the epidermis; the surface was usually covered by a layer of parakeratotic scales in lesions less than six months' duration, and by a hyperkeratotic layer in lesions of longer standing. Degenerative changes of the epidermis were observed in two lesions associated with secondary infection and surface ulceration.

TABLE 2

RESULTS OF DRUG-SUSCEPTIBILITY TESTS OF MYCOBACTERIUM BALNEI

Drug	Concentration of Drug (γ per ml.)			
	2	5	10	20
Isoniazid	+	±	0	0
Ethioniamide	0	0	0	0
Streptomycin	+	0	0	0
Neomycin	+	+	0	0
Kanamycin		±	0	0
Cycloserine		0	0	0
Pyrazinamide		+	+	+

+ = no inhibition; ± = partial or no inhibition.

The inflammatory changes in eight lesions of less than six months' standing were of a granulomatous character consisting mainly of an admixture of round cells, chiefly lymphocytes and epithelioid cells. Tubercle-like structures with Langhans' giant cells were observed in only three of these lesions (figure 3). In the others, the epithelioid cell infiltrates were more diffuse and not infrequently contained foci of "fibrinoid" necrosis, described by Linell and Nordén (5). The caseous type of necrosis commonly seen in true tuberculosis was not evident in any of the cases in this study. Nerve involvement was not observed.

The earliest lesion examined was of one month's duration and differed from all others of longer standing. The bulk of the lesion was composed of a focal area of polymorphonuclear leukocytes in the corium, and extended upward

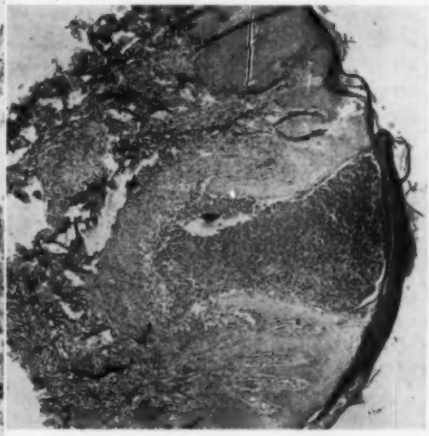

FIG. 3 (Left). A discrete tubercle-like structure with giant cells and areas of lymphocytic infiltration in the corium; three-month lesion. (×200) (U. S. Army photograph)

FIG. 4 (Right). Wedge-shaped area of polymorphonuclear leukocytes extending from corium through epithelium with an overlying layer of parakeratotic scales; one-month lesion. (×40) (U. S. Army photograph)

through the surface epithelium to a covering layer of parakeratotic scales (figure 4). The abscess was surrounded by a zone of rather large, pale-staining histiocytes which were encroaching on the purulent exudate. Acid-fast bacilli were readily demonstrable within several of the histiocytes. A pure culture of *M. balnei* was obtained from the untreated specimen notwithstanding the purulent character of the lesion.

There were seven lesions of six months' duration or more. In five the cellular reaction was of a nonspecific character consisting almost entirely of focal aggregates of lymphocytes in the corium, commonly situated about blood vessels and sweat glands. Tuberculoid changes were lacking, and these lesions were considered to be in the healing stages, only a residual round-cell infiltration remaining in a sclerotic corium (figure 5). The other two lesions in this group still contained residual foci of tubercle-like structures among the lymphocytic infiltrates.

Acid-fast bacilli were observed in tissue sections in 9 of the 15 cases. Six were also positive for acid-fast bacilli in direct microscopic examination. A positive culture was obtained in 12 of the 15 specimens studied microscopically. As far as could be determined, the bacilli seen in tissue sections were intracellular. They were somewhat longer and broader than true tubercle bacilli, and frequently showed crossbanding (figure 6). The lesions in which the acid-fast organisms were more readily seen in tissue sections also yielded greater numbers of colonies in culture. In the majority of the lesions, however, the bacilli were sparse and were found only after prolonged search.

Discussion

The isolation of *Mycobacterium balnei* from the skin lesions and the swimming pool in Glenwood Springs, Colorado, confirmed the finding of the Swedish investigators on the significance of *M. balnei* as the cause of "swimming pool granuloma," and proved its presence in this country. The same bacterium was also isolated recently from patients who had contracted skin lesions while swimming on beaches of the Hawaiian islands and in a swimming pool in Jacksonville, Florida.[7] Studies by Shepard (17), Bojalil (18), and the present writers indicate that the mycobacteria isolated in 1926 by Aronson (9) and in 1941 by Baker and Hagan (10) from diseased salt water fish and tropical fish kept in aquariums were similar to *M. balnei*. All of these findings suggest that *M. balnei* is a normal habitant of the soil, with a preference for humid soils or water of moderately warm temperature. The swimming pool in Glenwood Springs provided such conditions. It was an open-air hot-springs pool, built in 1892. The temperature of the water was about 30°C. The pool was not chlorinated because the addition of chlorine produced cloudiness of the water due to reaction with some components of the hot spring water.[8]

The history of this outbreak indicates that isolated cases of the disease were observed for several years before its source and cause were identified. The difficulties encountered in recognizing the true nature of the disease were due to the fact that the histopathologic picture of the lesions, as indicated by this study, often showed only the signs of a nonspecific chronic inflammatory process which could be produced by a variety of traumatic or infectious agents. In other cases in which tubercles, Langhans' giant cells, and acid-fast bacilli were seen, the process was considered a tuberculous infection, and this diagnosis seemed to be supported by the finding of a positive tuberculin reaction. It is clear, however, from the observations made in Sweden as well as in Colorado that infection with *M. balnei* also causes a positive tuberculin reaction.

In most cases it will be possible to make a definitive diagnosis of skin granuloma due to *M. balnei* from the site of the lesion, the past history of swimming, the positive tuberculin reaction, histologic examination and, most conclusively, the isolation of the organism.

Summary

Mycobacterium balnei was identified as the cause of an outbreak of "swimming pool granuloma" in Colorado, and it was isolated from 17 of 24 biopsy specimens. The methods for the isolation and identification of this bacterium are described and evaluated. The histopathology of the lesions and its diagnostic significance are discussed.

[7] Roche Report on Medical Progress, June 13, 1960, Vol. 2, No. 23.

[8] The pool has been completely reconstructed and no cases of the disease have since been observed.

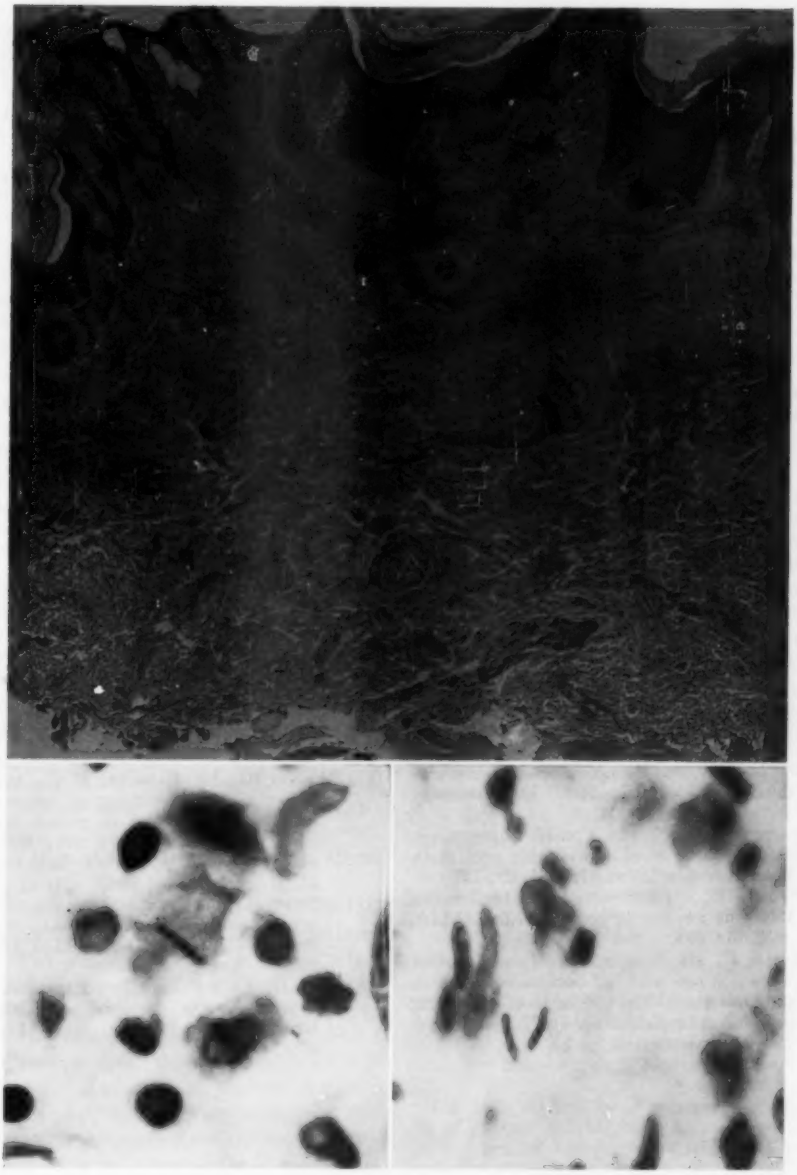

FIG. 5 (Upper). Perivascular foci of lymphocytes in a sclerotic corium; six-month lesion. (×40) (U. S. Army photograph)

FIG. 6 (Lower). Tissue section from three-month-old lesion showing acid-fast bacilli with more or less distinct "cross-barring." (×1800)

Sumario

Estudio Bacteriológico e Histopatológico del Granuloma Cutáneo Debido al Mycobacterium Balnei

El *Mycobacterium balnei* fué identificado como la causa de un brote de "granuloma de las piscinas" en el Estado de Colorado, aislándose de 17 de 24 ejemplares biópsicos. Se describen y justiprecian los métodos empleados para el aislamiento y la identificación de esta bacteria. También se discuten la histopatología de las lesiones y su importancia diagnóstica.

Resume

Une étude bactériologique et histopathologique d'un granulôme cutané dû à Mycobacterium balnei

On identifia *Mycobacterium balnei* comme la cause d'une épidemie de "granulome des piscines" au Colorado, et on l'isola à partir de 17 de 24 échantillons biopsiques. Les auteurs décrivent les méthodes employées pour isoler et identifier cette bactérie et pour l'évaluer. Les auteurs discutent l'histopathologie des lésions et leur signification diagnostique.

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EVALUATION OF THE AGAR-PLATE PRECIPITIN TEST FOR HISTOPLASMOSIS[1]

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INTRODUCTION

Histoplasmosis is one of the most prevalent of human pulmonary diseases. It has been estimated that in the United States 30,000,000 persons of the present population have been infected by *Histoplasma capsulatum* (1). Fortunately, most infections caused by this fungus are asymptomatic and do not require medical attention. When clinical signs and symptoms of the disease do appear, the establishment of a correct diagnosis is often a difficult medical problem. Clinically, histoplasmosis mimics other diseases to such a remarkable extent that diagnosis is often inconclusive unless supported by positive results of appropriate laboratory tests.

Serologic procedures of various types have proved to be very useful in finding suspected cases of histoplasmosis. For several years, the Kansas City Field Station has been conducting serologic surveys of patients in tuberculosis sanatoriums in various parts of the United States and Canada (2). These serologic surveys have been done to determine the prevalence of active histoplasmosis in tuberculosis sanatoriums and to find patients with proved active infections for inclusion in therapeutic studies. All serum specimens from these continuing surveys, about 14,000 annually, have been sent to the Microbiology Diagnostic Unit, Laboratory Branch of the Communicable Disease Center, Atlanta, Georgia. The complement-fixation test (3) with histoplasmin and yeast-phase antigens has been used exclusively in this work.

Although complement-fixation is the preferred serologic test for histoplasmosis, antigens for it are not widely available, and the test can only be done by well trained personnel. For this reason, tests for histoplasmosis are not generally performed in most laboratories, but require shipment of serum specimens to a central laboratory.

Among serologic procedures, the precipitin test is considered to be one of the simplest. Salvin and Furcolow (4) used a precipitin test in which sera were tested with antigen in capillary tubes. This procedure was evaluated by the Microbiology Diagnostic Unit of the Communicable Disease Center several years ago and found to have two serious limitations: (*1*) Some sera gave nonspecific reactions with the medium used to prepare the antigen; (*2*) lipemic sera could not be tested.

In 1949, Ouchterlony (5) described a modified form of the precipitin test for antigen characterization in agar plates.

Heiner (6) in 1958 employed an agar-gel procedure in which he utilized histoplasmin to produce a precipitin reaction. He stated that the development of either an *M* or an *H* band using purified antigens was sufficient evidence for a presumptive diagnosis of histoplasmosis if there had been no recent skin testing.

A modified version of the Heiner procedure was employed by the Microbiology Diagnostic Unit, and an evaluation of this test with the standard complement-fixation procedure was set up to determine its value as a rapid diagnostic procedure. The results of this evaluation study are reported here.

MATERIALS AND METHODS

Serologic tests: The complement-fixation procedure employed was the modified 50 per cent hemolysis end point method used by the Communicable Disease Center for more than ten years (3).

The agar gel employed was prepared with 0.9 gm. of sodium chloride, 0.4 gm. of sodium citrate, and 0.9 gm. of Special Agar-Noble (Difco)[2] dissolved in 100 ml. of distilled water. Melted phenol (0.25 ml.) was added as a preservative. After autoclaving at 120°C. for ten minutes, the final pH was 6.7.

Noble's agar was chosen since other types of

[1] From the Communicable Disease Center, Bureau of State Services, Public Health Service, U. S Department of Health, Education, and Welfare, Atlanta, Georgia; and the Communicable Disease Center, Kansas City Field Station, Kansas City, Kansas.

[2] The use of trade names does not constitute endorsement by the U. S. Public Health Service.

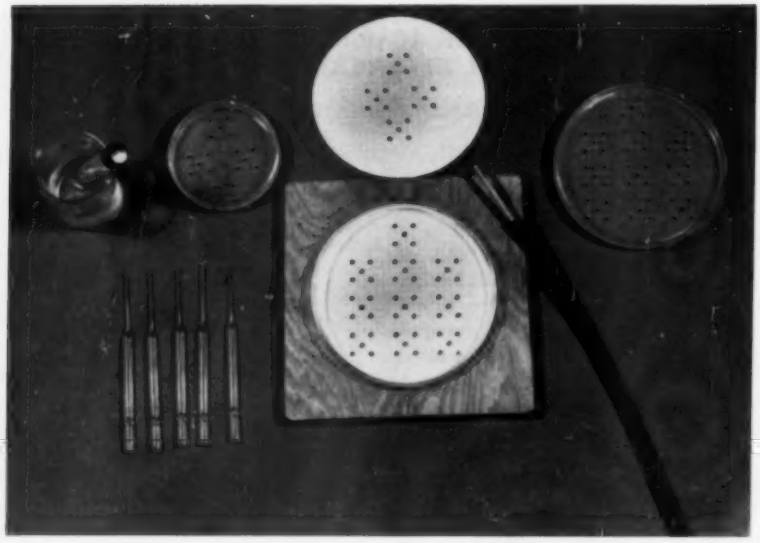

FIG. 1. Preparation of agar plates. The serum wells were 15 mm. apart, whereas the central antigen well was 10 mm. from each serum well.

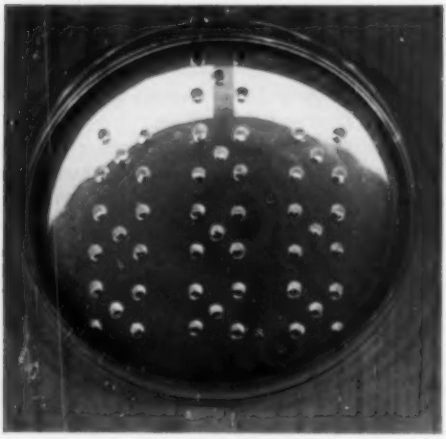

FIG. 2. Agar plate reading box.

agar-agar were less translucent. The sodium citrate was used to control the pH and was found to reduce halo formation around the serum wells.

Ninety-five milliliters of the melted agar were poured into 130-mm. diameter Petri dishes, or 40 ml. of agar into 90-mm. Petri dishes. The plates were placed on a level surface on which the agar was allowed to harden. The wells, arranged according to the pattern of a template, were cut into the agar with a 3/16-inch hollow aluminum tube connected to a vacuum line (figure 1). The bottom of each well was then sealed with a drop of agar. The pattern employed permitted 40 serum specimens to be tested in the large plate and 12 in the standard size plate. Approximately 0.08 ml. of serum or antigen was added to the wells by means of capillary pipettes. The precipitin reaction occurred between the antigen and antibody which diffused from the wells in the agar.

A simple viewing box was constructed to facilitate reading of the reactions. It contained a 22-watt 8-inch circular fluorescent tube mounted two inches below the top of the box. A black background was placed beneath the fluorescent tube to provide contrast (figure 2).

Antigens: Two types of antigens were required for the study. Histoplasmin was obtained by growing four strains of *H. capsulatum* (105, A28, A228, A232) separately in modified tuberculin medium of Smith *et al.* (7) for six months. After the addition of 1:10,000 merthiolate and storage for three days at room temperature, the broths were filtered and pooled in equal amounts. This histoplasmin was used in the complement-fixation test at a 1:32 dilution, as determined by a box titration of a control serum. For the agar plate precipitin test, undiluted histoplasmin was concentrated ten times by perevaporation in a dialysis bag. A fivefold concentration of the histoplasmin antigen was selected for the precipitin test on the basis of visibility of precipitin lines with various dilutions of the concentrate on 20 serum specimens positive by the complement-fixation test.

Two yeast-phase antigens were produced from

strain A-811 of *H. capsulatum*. A 10 per cent stock antigen obtained from yeast-phase cells grown in BHI agar at 37°C., diluted 1:32, was employed for the yeast-phase complement-fixation test. A soluble precipitin antigen was prepared according to Salvin's (8) method.[5] The yeast-phase precipitin antigen as shown in the footnote was discarded in favor of the histoplasmin concentrate.

The development of one or more thin opaque lines of precipitation between the antigen and serum wells was regarded as a positive reaction.

No attempt was made to report titers with precipitin-positive serum specimens, since these were lower than the titers in the complement-fixation test. It was found that 80 per cent of the positive serum specimens reacted in twenty-four hours, 93 per cent in forty-eight hours, and 100 per cent in seventy-two hours. Incubation was performed at 20°C.

Serum specimens: The test serum specimens were obtained from a number of state sanatoriums over a six-month period. A group of 5,211 were tested with both complement-fixation antigens and with the agar-plate precipitin test.

Results

The results of the agar-plate precipitin test compared with the complement-fixation test on serum specimens positive with histoplasmin or yeast-phase antigen are shown in table 1. The results indicated that the proportion of serum positive with the precipitin test increased as the titer for both the histoplasmin and yeast-phase antigens rose. There was better agreement between the precipitin test and the histoplasmin complement-fixation results than with the yeast-phase complement-fixation. This was particularly noticeable with yeast titers of 1:8 to 1:16. Eighty-three per cent of the histoplasmin complement-fixation positive serum specimens were positive with the agar-plate test, but only 14 per cent were positive with the yeast complement-fixation positive serum.

When the histoplasmin and the yeast-phase complement-fixation antigens were both positive (table 2), 84 per cent were positive with the agar-plate test. This agreement was greater than 90 per cent when the histoplasmin and complement-fixation yeast titers were 1:16 or higher.

The results of the agar-plate test on serum specimens negative by histoplasmin and yeast-phase antigens are shown in table 3.

[5] In a preliminary trial, 16 of 52 complement-fixation positive serum specimens gave positive agar-plate precipitin reactions with the histoplasmin antigen. In contrast, the soluble yeast precipitin antigen was positive in only 3 of the 16 serum specimens and negative with the remaining histoplasmin-negative serum specimens.

TABLE 1

Results of the Agar-Plate Precipitin Test on Serum Specimens Which Were Positive with Only One of the Complement-Fixation Antigens

Positive Serum Specimens with Histoplasmin Complement Fixation Only			Positive Serum Specimens with Yeast Complement Fixation Only		
Titer to Histoplasmin Complement Fixation	Number Tested	Per Cent Plate Precipitin Test Positive	Titer to Yeast Complement Fixation	Number Tested	Per Cent Plate Precipitin Test Positive
1:8	37	70	1:8	249	6
1:16	26	92	1:16	160	7
1:32+	38	88	1:32+	133	29
Total	101	83	Total	542	14

TABLE 2

Comparison of the Agar-Plate Precipitin Test on Sera Complement-Fixation Positive with Both Histoplasmin and Yeast-Phase Antigen

Yeast Complement Fixation Titers	Histoplasmin Complement Fixation					
	1:8		1:16		1:32+	
	Number	Per Cent + Plate Precipitin Test	Number	Per Cent + Plate Precipitin Test	Number	Per Cent + Plate Precipitin Test
1:8	20	65	21	80	13	100
1:16	37	57	23	91	11	100
1:32+	38	70	31	96	94	98

288 total positive complement-fixation agar gel + 84 per cent.

TABLE 3

Results of the Agar-Plate Precipitin Test on Complement-Fixation Negative Sera

	Number	Agar Gel Positive
		per cent
Negative both complement-fixation antigens	4,280	1.2
Negative histoplasmin complement-fixation antigen	4,822	2.5
Negative yeast complement-fixation antigen	4,381	2.7

The agar-plate test was positive with 1 per cent of the serum specimens negative with both complement-fixation antigens. A 1 per cent increase of agar-plate positive reactions was ob-

TABLE 4

REPRODUCIBILITY OF RESULTS WITH THE AGAR-PLATE PRECIPITIN TEST IN ATLANTA AND KANSAS CITY AND COMPARISON OF PRECIPITIN WITH THE COMPLEMENT-FIXATION RESULTS

Complement-Fixation Results	Both Laboratories		Nonagreement Both Laboratories	Number Tested	Per Cent Agreement
	PPT+	PPT−			
H & Y+	225	31	29	285	90
H+	73	15	9	97	91
Y+	65	433	34	532	94
H & Y−	51	3,833	98	3,982	98
Total	414	4,312	170	4,896	96

H = Histoplasmin.
Y = Yeast phase.
PPT = Plate precipitin test.

tained on serum specimens in which only one of the complement-fixation antigens was negative.

The reproducibility of the agar-plate precipitin test was tested at the Histoplasmosis Investigation Laboratory in Kansas City and in the Microbiology Diagnostic Unit. The sera were first tested in the former laboratory with the antigen and the method used by the Microbiology Diagnostic Unit. The sera then were shipped to Atlanta where complement-fixation tests and the agar-plate precipitin test were performed. The results are shown in table 4.

The Atlanta and Kansas City Laboratories showed 96 per cent agreement with the agar plate precipitin test on all 4,726 serum specimens tested. About 90 per cent agreement was obtained when only the complement-fixation positive serum specimens were compared.

DISCUSSION

The presence of positive reactions to serologic tests for histoplasmosis in different tuberculosis sanatoriums in the United States and Canada, as shown by Walls and associates (2), indicates that exposure to the causative organism occurs in a wide area. An easily performed, rapid serologic procedure would make possible the selection of patients for more thorough examination for histoplasmosis in the local endemic area.

The agar-plate precipitin procedure has properties that are desirable for the screening of patients for possible infection by *H. capsulatum*. The test is easily performed, economical, and lends itself to use by laboratories of limited resources.

These data do not answer the question of how many of the serum specimens positive by serology come from active cases of histoplasmosis. Data obtained by Brasher and Larsh (9) indicate that the isolation of *H. capsulatum* is greater among patients with higher complement-fixation titers. In this study it was found that the number of positive precipitin reactions increased with the serum specimens having high complement-fixation titers. It follows then that the agar-plate test can be used for the rapid identification of those patients on whom fungous isolation should be attempted.

Although the present precipitin antigen produced *M* and *H* bands according to Heiner (10), the number of bands is not considered significant for diagnostic purposes. More than one band was not obtained with low-titer complement-fixation serum. The antigen used in this study was prepared in the same manner as that used by Heiner, namely perevaporation but, in the writers' opinion, this does not constitute a "purified" antigen. From this it is believed that a presumptive diagnosis of histoplasmosis could also be made when only one clearly visible band was present.

Cross reactions are generally expected to occur between antigens for histoplasmosis and other fungal diseases. However, the agar-plate histoplasmin precipitin test gave no reaction with serum specimens from 14 proved cases of coccidioidomycosis in humans with complement-fixation titers of 1:16 to 1:256. Serum specimens from other proved fungal diseases such as blastomycosis were not available for comparison.

SUMMARY

An agar-plate precipitin test for histoplasmosis was studied on 5,211 specimens of human serum. A fivefold concentrate of histoplasmin was used as antigen. Precipitin tests were read as positive or negative within seventy-two hours. The lines or bands were clearly visible and easily read with a specially designed reading box. Two laboratories performed the agar-plate precipitin test on 4,482 serum specimens and obtained 96 per cent agreement in results. The agar-plate precipitin test can result in a presumptive diagnosis of histoplasmosis on which the need for

further diagnostic study of the patient can be based.

Sumario

Justipreciación de la Precipitinorreación con Agar-Placa para Histoplasmosis

En 5,211 ejemplares de suero humano se ha estudiado una precipitinorreación en agar-placa para la histoplasmosis. Como antígeno se usó histoplasmina concentrada al quíntuplo. Las precipitinorreacciones se leyeron como positivas o negativas en término de setenta y dos horas. Las líneas o franjas eran visibles claramente y legibles fácilmente con cajita de lectura diseñada *ad hoc*. Dos laboratorios ejecutaron la precipitinorreacción de agar-placa en 4,482 ejemplares de suero, obteniendo un acuerdo de 96 por ciento en los resultados. La precipitinorreacción de agar-placa puede dar por resultado un diagnóstico presuntivo de histoplasmosis en el cual puede basarse la necesidad de estudio diagnóstico ulterior del enfermo.

Resume

Evaluation du test à la précipitine sur plaque d'agar-agar dans l'histoplasmose

Les auteurs étudièrent un test à la précipitine sur plaque d'agar-agar dans l'histoplasmose, sur 5.211 échantillons de sérums humains. Un concentré—cinq fois—d'histoplasmine fut utilisé comme antigène. Les tests à la précipitine furent interprêtés comme positifs ou négatifs dans les soixante-douze heures. Les lignes ou les bandes furent clairement visibles et faciles à lire avec une boite d'interprétation faite spécialement à ce dessein. Deux laboratoires pratiquèrent le test à la précipitine sur plaque d'agar-agar sur 4.482 échantillons de sérum et ils obtinrent 96% d'accord dans les résultats. Le test à la précipitine sur plaque d'agar-agar peut fournir un diagnostic de présomption d'histoplasmose sur lequel on peut baser le besoin d'une étude diagnostique plus poussée du malade.

Acknowledgment

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THE EFFECT OF FILTRATION ON BIOLOGIC PRODUCTS[1,2]

II. Influence on Catalase and Lipase Activity

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INTRODUCTION

While examining the catalase activity of sonic-treated mycobacteria, it was noted that sterilization of small samples by Seitz filtration resulted in a complete loss of activity. This observation could not be attributed to the removal of intact organisms or cellular debris. Therefore, the present studies were undertaken to determine quantitatively the effect of filtration on certain enzymes from mycobacterial and mammalian sources. A previous communication has reported the effect of filtration on the serologic activity of Old Tuberculin (1).

MATERIALS AND METHODS

Filters. Seitz bacterial filters 35 mm. in diameter, fritted glass ultrafine 60 mm. in diameter, and millipore-type HA filters 47 mm. in diameter were employed. The filters had surface areas of 9.6, 28.3 and 17.3 sq. cm., respectively. Filtrations were carried out at room temperature (25°C.) under a negative pressure produced by means of a water aspirator adjusted to give a maximal pressure differential of about 70 cm. of mercury. The influence of pressure differentials on the removal of enzyme protein by the filters was determined in one series of experiments by the use of a Cenco-Pressovac electric pump, which produces an ultimate vacuum of 0.1 mm. of mercury or less.

Enzyme preparations. Mammalian: Crude bovine liver catalase, lyophilized bovine liver catalase (40 to 50 milliequivalents of sodium perborate), and lipase (steapsin) were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The enzyme solutions were prepared just prior to use by dissolving in 0.1 M potassium phosphate buffer, pH 7.0, except in certain experiments in which saline or phosphate-buffered saline was the diluent. Any undissolved residue was removed by filtration through two thicknesses of Whatman No. 40 filter paper. The enzyme solutions were then adjusted to give the desired activity, and hereafter will be referred to as "nonfiltered."

Mycobacterial: Crude extracts with catalase and lipase activity were prepared from *Mycobacterium phlei* and the H37Rv strain of *M. tuberculosis* by sonic oscillation of washed cells, suspended in phosphate buffer, in a 9 KC Raytheon oscillator for one hour. The bulk of the intact cells and cellular debris was removed by centrifugation before sterilization by filtration.

Nitrogen content. The concentration of total nitrogen in the enzyme preparations was determined before and after filtration by the method of Miller and Miller (2), which is suitable for analysis of samples with low nitrogen content.

Determination of enzyme activity. Catalase: Five milliliters of 0.1 M potassium phosphate buffer, pH 7.0, and 1 ml. of enzyme preparation, filtered or nonfiltered, were mixed and chilled to 4°C. At zero minutes, 5 ml. of prechilled 0.1 M hydrogen peroxide were added to the buffer and enzyme. The reaction was allowed to proceed at 4°C. for an appropriate time interval, and was stopped by the addition of 5 ml. of 20 per cent sulfuric acid. Each test included controls which contained heat-inactivated enzyme and others which were inactivated at zero minutes by the acid. Unused hydrogen peroxide was measured titrimetrically with 0.05 N potassium permanganate.

Lipase: Quantitative determinations of lipase activity were made using, essentially, the technique of Davis (3) for the estimation of small amounts of fatty acids in the presence of esters of these acids. Five milliliters of 10 per cent Tween® 80[3] in buffer and 5 ml. of lipase, filtered or nonfiltered, were allowed to react for two hours at 25°C. with occasional shaking. The liberated oleic acid was extracted and titrated.

The presence or absence of lipase activity was also shown by a simple but sensitive indirect bioassay method. Incubation of a source of lipase with Tween 80 in a medium without protective protein, such as bovine albumin, provides a more sensitive assay for lipase activity than most available chemical methods (4). This procedure was used to compare the effects of the microbial filters on this enzyme in mycobacterial extracts. Each filtrate to be tested was added to a sterile medium containing 0.05 per cent of Tween 80, but without protein. The concentration of enzyme solution ranged from 1 to 20 per cent in a total volume of 5 or 10 ml. Comparable controls were diluted with buffer. The tubes were then inoculated with a human strain of tubercle bacilli and incubated at 37°C., usually for

[1] From the Trudeau Laboratory of the Trudeau Foundation, Inc., Saranac Lake, New York.

[2] This study was supported by research grants E36C12 and E36C13 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service.

[3] Tween® 80 is the trade name of polyoxyethylene sorbitan monooleate, produced by the Atlas Powder Company, Wilmington, Delaware.

fourteen days. The rate of growth was measured in a Bausch and Lomb Spectronic 20 at 600 mμ against a blank of uninoculated medium. Hydrolysis of Tween 80 results in the formation of free oleic acid which is toxic at low concentrations for some strains of mycobacteria. Under the conditions used, inhibition of growth of the inoculum was indicative of lipase activity.

Results

Crude bovine catalase. Twenty-five–milliliter aliquots of crude bovine liver catalase solution were passed through the Seitz, fritted glass, and millipore filters at a differential pressure of 70 cm. of mercury. Upon completion of filtration, the filtrate was removed from the flask and another 25-ml. aliquot was added until a total of 100 ml. had been filtered through each type of filter. The amount of hydrogen peroxide decomposed in the presence of each fraction was then determined at various time intervals and compared with that remaining in control systems containing nonfiltered enzyme. The average percentage of hydrogen peroxide acted upon in ten minutes at 4°C. is given in table 1. The per cent of activity lost in the tables is based on the amount of substrate decomposed by the nonfiltered catalase taken as 100 per cent activity.

TABLE 1

Effect of Filtration of 25-Ml. Aliquots on Crude Bovine Catalase Activity

Filter	Aliquot	Hydrogen Peroxide Decomposed	Activity Lost*
		per cent	*per cent*
Nonfiltered control		79.5	
Seitz	1	0	100.0
Seitz	2	0	100.0
Seitz	3	0	100.0
Seitz	4	0	100.0
Millipore	1	13.5	83.0
Millipore	2	65.0	18.2
Millipore	3	76.6	3.6
Millipore	4	78.2	1.6
Fritted glass	1	0	100.0
Fritted glass	2	0.2	99.7
Fritted glass	3	1.5	98.1
Fritted glass	4	3.6	95.5

* If per cent H_2O_2 decomposed by nonfiltered enzyme (a) ∝ 100 per cent activity (b), then per cent H_2O_2 decomposed by filtrate (c) ∝ ? per cent activity (x): x = bc/a and per cent activity lost = b − x.

It will be noted in table 1 that considerable activity was lost in the first millipore-filtered aliquot, but reduction was negligible in the third and fourth fractions. Additional tests showed that filtration of 100 ml. of equivalent enzyme solution in a continuous flow resulted in a decrease in activity of 17 per cent in the millipore filtrate. This loss was apparently due to a retention by the coarse fritted-glass base of the filter assembly, since a comparable dec[illegible]e was found in the absence of the millipore m[illegible]rane.

The Seitz filtrates were inactive, [illegible]d prior washing of the Seitz disc with salin[illegible] buffer had no effect. Attempts to elute the enzyme from the disc were unsuccessful; however, retention of catalase by the Seitz was demonstrated qualitatively by adding the substrate to the filter cup. Even after the disc had been washed copiously by the passage of saline or buffer there was a vigorous evolution of oxygen upon the addition of hydrogen peroxide.

When 25-, 50-, 75-, 100-, and 200-ml. amounts of crude bovine catalase solution were sterilized through different Seitz filters, only the 200-ml. fraction possessed activity, and this was of a low degree. However, the results indicate the adsorptive capacity of the filter.

As shown in table 1, virtually all catalase activity was removed from the four successive 25-ml. aliquots by the ultrafine fritted-glass filter. Even a fritted-glass filter of coarse porosity significantly lowered the enzyme concentration of samples less than 100 ml. In view of these findings, the efficacy of crude catalase, sterilized by filtration through ultrafine fritted glass, in media for the cultivation of isoniazid-resistant, catalase-negative tubercle bacilli seemed questionable. A stock solution of 1 mg. per ml. in saline sterilized in this manner was recommended by Middlebrook and associates (5) for use in their 7H-10 medium. This is at least four to eight times more concentrated than the enzyme solutions employed in the experiments already described in this report. Therefore, 25 ml. of 1 mg. of crude bovine liver catalase per ml. of saline were filtered through ultrafine fritted glass as well as Seitz filters at the standard pressure differential provided by the water pump. None of the substrate was decomposed by either filtrate during the time required by the nonfiltered ma-

TABLE 2

ACTIVITY OF 1 MG. OF CRUDE CATALASE PER ML. OF SALINE AFTER STERILIZATION BY FILTRATION

Filter	Hydrogen Peroxide Decomposed	Activity Lost	N per Ml.
	per cent	*per cent*	γ
Nonfiltered control	90.0		104.6
Fritted glass	0	100.0	15.8
Seitz	0	100.0	14.8
Millipore	81.6	9.3	84.3

TABLE 3

ACTIVITY OF A 1:10 DILUTION OF 1 MG. OF LYOPHILIZED BOVINE CATALASE PER ML. OF BUFFERED SALINE AFTER STERILIZATION BY FILTRATION

Filter	Hydrogen Peroxide Decomposed	Activity Lost	N per Ml.
	per cent	*per cent*	γ
Nonfiltered control	99.5		13.8
Fritted glass	12.8	87.1	7.3
Seitz	99.0	0.5	8.5
Millipore	99.5	0	11.7

TABLE 4

EFFECT OF FILTRATION ON MYCOBACTERIAL CATALASE ACTIVITY

Filter	Hydrogen Peroxide Decomposed	Activity Lost	N per Ml.
	per cent	*per cent*	γ
Nonfiltered control	80.4		65.0
Millipore	72.3	10.1	30.3
Fritted glass	51.3	36.2	23.2
Seitz	0	100.0	17.4

terial to react with 90 per cent of the peroxide (table 2).

Under the same conditions of testing, the millipore filtrate retained considerable activity, as in the case of less concentrated material. Analysis of the nitrogen content before and after filtration revealed corresponding, but not comparable, reductions.

A discrepancy in the activity of saline-catalase solutions after filtration through fritted glass might be due to a difference in the vacuum applied. This possibility was investigated, but it was found that the greater vacuum provided by the electric pump did not produce active Seitz and fritted-glass filtrates. However, using phosphate buffer as the diluent, the enzyme was not removed completely by either filter under the influence of the stronger vacuum although the rate of flow was not significantly increased, the activity apparently being related to ion concentration.

Lyophilized bovine catalase. These results prompted a study of the effects of sterilizing filters on a more refined catalase preparation, lyophilized beef-liver catalase dissolved in buffered saline, which was the type originally suggested for use in culture media for tubercle bacilli (6). Millipore and Seitz filtrates of 25-ml. aliquots of 1 mg. per ml. were highly active, as may be seen in table 3. However, only a fraction of the enzyme was recovered from the fritted-glass filter. The difference was shown by diluting the nonfiltered enzyme and the filtrates equivalently, so that the entire reaction time of one minute was required to decompose the substrate in the controls.

Mycobacterial catalase. The effects of microbial filters on the breakdown of hydrogen peroxide by mycobacterial extracts at 4°C. in ten minutes are shown in table 4, giving the average values of activity from a representative experiment in which 15-ml. aliquots of a crude sonic extract of *M. phlei* were filtered.

All detectable catalase activity was removed from the limited volume of extract by Seitz filtration, in common with dilute mammalian catalase. Appreciable activity was present in the fritted-glass filtrate, but the membrane filter was superior under the conditions of the study. A highly active preparation from *M. phlei*, which was passed through a Seitz filter in small aliquots and sampled at intervals, became progressively more active after 20 ml. had been filtered, again demonstrating the adsorptive capacity of the filter.

Sonic extracts of the H37Rv strain possessed less activity than those prepared from the saprophyte, but the enzyme of the virulent organism was affected proportionately by the three filters.

Pancreatic lipase. A 15-ml. aliquot of 0.5 per cent steapsin was found to pass through the membrane filter with about one-fourth reduction, while more than half was retained by the fritted-glass filter. Virtually all of the enzyme

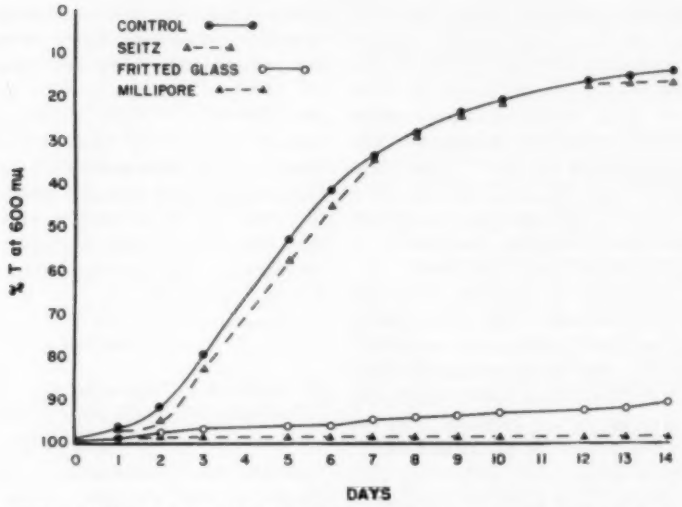

FIG. 1. Effect of filtration on inhibition of H37Rv due to lipase activity of *M. phlei* extract.

was removed by the Seitz filter, thus supporting an observation by Vogel (7).

Mycobacterial lipase. In a study of lipase in mycobacteria (8), repeated experiments have shown that most of the enzyme can be recovered if the crude extracts are sterilized by millipore filtration, but that no detectable activity is present after Seitz filtration up to 50 ml.

In figure 1, inhibition of growth of the H37Rv strain reflects the presence of free oleic acid formed as a result of enzymic hydrolysis of Tween 80 by a 10 per cent extract of *M. phlei*, filtered in 15-ml. volumes. Before filtration, the sonicate, containing some intact cells, had a nitrogen value of 50.5 γ per ml. The millipore, fritted-glass, and Seitz filtrates gave values of 17.8, 16.7, and 15.4 γ per ml., respectively. Results with fritted-glass filtrates were variable, and only slight inhibition was noted with these in many instances.

Using an extract of the H37Rv strain as a source of lipase, 92 per cent of its bacteriostatic activity in the Tween-synthetic medium was removed by Seitz filtration compared with the millipore-filtered material.

DISCUSSION

In the present study the millipore filter, in general, had the least effect on the two enzymes studied, regardless of source. This is not surprising since retention by the membrane is dependent upon pore size. However, this filter—as is the case of all filters—must be restricted to fluids which do not alter it. The adsorptive capacity of the Seitz filter limits its value in the sterilization of small volumes of some biologic preparations, but may prove useful in differential filtration.

Concentration, volume, solvent of the enzyme, and adsorption on glass and asbestos were, as anticipated, important factors in determining the activity of the filtrates. Adsorption of the enzyme proteins was nonspecific but rather consistent in degree for each filter in replicate experiments, except in the case of fritted-glass filters. No relationship was noted between activity and rate of filtration or surface area of the filters.

It is understood that the quantitative data reported here apply only to the preparations studied and under the experimental conditions outlined. Admittedly, many biologic materials may not be affected significantly by filtration under specified conditions. However, decontamination by filtration has become so routine in the laboratory that many investigators fail to examine critically this step in a procedure. For example, the inclusion of catalase in media for the culti-

vation of isoniazid-resistant, catalase-negative tubercle bacilli has been recommended. Apparently, the experimental work supporting the use of this enzyme to promote the growth of these organisms was done with lyophilized bovine catalase in buffered saline (6). Subsequent published methods advocate the use of *crude* catalase. Since only 2 ml. (5) to 3 ml. (9) of a 1-mg. per ml. solution of the crude enzyme are prescribed per liter of medium, sterilization of 25 ml. through a fritted-glass filter seems a liberal amount to prepare at one time. However, under these conditions and using widely different vacuums, the filtrate contains no demonstrable catalase activity by the permanganate-titration method when the 60-mm. filter is employed. There is no mention in the literature that the catalase solutions should be pretreated before filtration through fritted glass. But unless they are first put through filter paper or centrifuged, even small volumes will not pass through the 20- and 30-mm. size filters within a reasonable period of time, whereas they will pass through the 60-mm. filter. For that reason or because the larger filter happens to be the most accessible, many laboratory personnel may unwittingly remove much or all of the enzyme's activity by the filtration procedure.

Davis and Dubos (4) suggested that bovine albumin to be used in media with Tween 80 be heated to 56°C. for thirty minutes to destroy the small amount of lipase present. The albumin which they tested had been sterilized by ultrafine fritted-glass filters. The removal of mammalian lipase by Seitz filtration, as shown in this report, indicates that inactivation of the albumin may be an unnecessary step when these filters are used. However, this has not yet been tested. On the other hand, it should be kept in mind that a protein solution which has been sterilized by a highly adsorptive filter will be reduced in concentration, and a basal medium which has been fortified with this filtrate will not be the same as one to which the filtrate of a less retentive filter has been added.

Summary

This report emphasizes the importance of selecting a suitable filter for the sterilization of limited quantities of biologic products so that undesirable changes in content, which may be avoidable, do not result from the procedure. The effects of millipore type HA, bacterial Seitz, and ultra-fine fritted-glass filters on mycobacterial and mammalian catalase and lipase were studied. In general, using a maximal standard pressure differential of 70 cm. of mercury, small volumes of either enzyme from both sources in low concentrations were completely inactivated by Seitz filtration but were not greatly affected by the membrane filter. Results with the fritted-glass filter were variable. Corresponding, but not comparable, reductions in nitrogen content after filtration were shown.

Sumario

El Efecto de la Filtración sobre los Productos Biológicos. II. Influjo sobre la Actividad de la Catalasa y la Lipasa

Recalca esta comunicación la importancia de escoger un filtro adecuado para la esterilización de cantidades limitadas de productos biológicos a fin de que no deriven del procedimiento alteraciones contraproducentes del contenido, que pueden evitarse. Se han estudiado los efectos de los filtros miliporosos tipo HA, bacterianos de Seitz y vitrificados ultrafinos sobre la catalasa y la lipasa micobacterianas y de mamífero. En general, usando una presión diferencial estándard máxima de 70 cm. de mercurio, se inactivaron totalmente pequeños volúmenes de una u otra enzima de ambas procedencias a concentraciones bajas, con la filtración Seitz, pero no se afectaron mayor cosa con el filtro de membrana. Los resultados con el filtro vitrificado fueron variables. Se observaron reducciones correspondientes, pero no comparables, del nitrógeno después de la filtración.

Resume

L'effet de la filtration de produits biologiques. II. Influence sur l'activité de la lipase et de la catalase

Ce rapport signale l'importance du choix d'un filtre convenable pour la stérilisation de quantités limitées de produits biologiques de sorte que des changements indésirables du contenu, qui peuvent être évités, ne résultent pas du procédé. Les auteurs étudièrent les effets de filtres milliporeux de type HA, de filtres bactériens de Seitz, et de filtres ultrafins de verre pilé sur la catalase et la lipase de microbactéries et de mammifères. En général, en utilisant une pression différentielle maxima standard de 70 cm de mercure, des petits volumes de l'un des enzymes des deux sources furent complètement inactivés à des concentra-

tions faibles, par la filtration de Seitz, mais ne furent pas beaucoup affectés par la membrane filtrante. Les résultats avec le filtre de verre pilé furent variables. Après filtration, il apparut des réductions du contenu azoté, lesquelles correspondaint mais n'étaient pas comparables.

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FIXATION OF THE LUNG BY FORMALIN STEAM IN A CONTROLLED STATE OF AIR INFLATION[1, 2]

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Introduction

In the course of a quantitative morphologic study on architecture and dimensions of the human lung (1), the need occurred to fix cadaver lungs in a controlled degree of air inflation. Recently, several methods have been presented which allow the fixation of lungs in an expanded state by means of compressed air (2) or formaldehyde in the state of vapor or fume (3, 4). These methods are quite time-consuming and have the disadvantage of providing a dry lung. The extraction of water from the delicate tissues of the lung without replacement by another fluid results in considerable shrinkage and distortion of the membranous structures. Blood vessels, for example, appear as flat ribbons. Moreover, the histologic quality of a specimen dried without previous rapid freezing is generally bad.

Fixation with vaporous media has several advantages over the conventional instillation of 4 per cent formaldehyde solution into the bronchial tree (5–7). Vaporous media appear more "physiologic" in maintaining the inflation of the airways and are less likely to induce distortion artefacts. With fluid instillation the normal shrinkage of alveolar septa induced by formaldehyde has to take place over an incompressible fluid core, thus leading to distortion of the relative dimensions with danger of flattening of hollow structures such as blood vessels. Additional distortion may result from external compression by immersing the heavy lungs in fluid.

The method for fixation of lungs by formalin steam presented here was developed to satisfy the following conditions: (*a*) the lungs should be fixed in a controlled state of air inflation; (*b*) tissue shrinkage induced by formaldehyde should be allowed without restriction; however, it should be possible to determine the degree of shrinkage quantitatively; (*c*) the fine structure of the tissue should be well preserved; (*d*) the method should be rapid.

Method and Material

The general arrangement of the apparatus used to apply formalin steam to the lungs is illustrated in the diagram of figure 1.

The lung is mounted in a Lucite box (*N*) with the bronchus open to the exterior at *C*. The side walls of the box are provided with several holes which may be used for various purposes, e.g., for vascular injections if desired. One hole is provided with a manometer (*F*); through another one (*E*) air is sucked out of the box with an air pump (*J*). The air is passed through water in the flask (*G*) in order to remove formalin fumes. The negative pressure thus created in the box (*N*) can be regulated easily with a screw clamp at the side arm (*H*). The cover (*O*) of the box does not need to be sealed on tightly since the negative pressure in (*N*) is controlled by a flow of air. This arrangement allows the lung to be inflated with negative "pleural" pressure to any desired extent. It also allows the lung to be "breathed" by changing the resistance of the side arm (*H*) as needed.

Formalin steam is applied to the lung through the airways by means of the apparatus to the right of the box. A 3,000-ml. flask (*A*) is filled with approximately 1,500 ml. of a solution of 2 parts 37 per cent formaldehyde and 1 part of water, which is heated by a Bunsen burner. The steam escapes through a single opening in a rubber-stopper and passes through a T-tube (*B*), whose side arm is immersed in water (*M*) to a depth of 5 to 10 cm. This side arm acts as a pressure escape valve, keeping the steam pressure constant. At the same time formaldehyde fumes escaping through this valve are absorbed by the water which is constantly exchanged by inflow of fresh water through (*K*) and drainage through the syphon (*L*).

The steam is led into the bronchial tree through the T-tube (*C*), which is fitted onto the connectors (*Cc*). The side arm (*D*) and clamp (*P*) allow inflation of the lung with room air after the equip-

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[2] Supported by a research grant from the New York Heart Association; by Grant H-2001 from the National Heart Institute, National Institutes of Health; and by the Office of Naval Research under contract with Yale University.

[3] Former Research Fellows in the Department of Pathology, Yale University School of Medicine.

[4] Present address: Cardiopulmonary Laboratory, First Medical and Chest Services (Columbia University Division), Bellevue Hospital, New York 16, N.Y.

ment has been completely assembled and before the formalin steam is allowed to enter the lung.

The connector (*C*) is constructed according to the recommendations of Moolten (7) and Liebow and associates (8). It consists of two short lengths of threaded metal pipe (*b,e*) and two corresponding sleeves (*c,d*). The pipe (*b*) is fitted tightly into a rubber stopper (*a*). On both ends of (*b*), the sleeves (*c,d*) are tightly screwed on. The pipe (*e*) is used to cannulate the trachea or bronchus and is secondarily screwed into the lower sleeve (*d*). A piece of rubber tubing is fitted around the conical end of the glass T-tube (*Cf*); this can then be tightly inserted into the opening of sleeve (*c*).

The lung is carefully removed from the chest, avoiding cuts into the pleura and leaving as long a piece of the main bronchus as possible. (The usual practice is to fix only one lung with this procedure; however, it is possible to fix both lungs, if they are properly mounted.) The pleura has to be examined carefully for tears and cuts. These have to be either sewn over tightly or ligated, depending on their size. Small light-weight spring-loaded paper clips render good service for clamping off longer cuts, which cannot be tied off easily. If major cuts are left unclosed, uneven inflation and hence uneven fixation will result.

The metal pipe (*Ce*) is tied into the bronchus. If injection of the blood vessels is desired, these should be cannulated at this point. Otherwise, it is advisable to tie off the major branches in order to avoid exsanguination. If possible, this should be done *in situ* before transection of the vessels. The free end of the pipe (*Ce*) is then screwed into the sleeve (*Cd*). Before lowering the lung into the box, the pleural surface is lightly painted with some glycerin diluted with water on a cotton swab; this will prevent excessive drying of the surface under the influence of the heat.

The apparatus is assembled as shown in figure 1, the clamp (*P*) is closed, and the side arm (*D*) is open. The lung is then inflated with room air by rhythmically varying the negative pressure at (*H*) until the whole lung is well inflated. The degree of inflation can be checked with the manometer *F*. It has been found convenient, however, to inflate the lung gradually until the maximal degree of inflation is almost reached, i.e., until a change in pressure causes only a slight change in volume. The pressure is then slightly reduced and the degree of inflation is maintained constant. This state of inflation should correspond approximately to three-fourths maximal inflation (*see* below).

The heating flask (*A*) is now started, clamp (*P*) still being closed. The contour of the expanded lung is drawn with a marking pencil onto the front panel of the box (*N*) as an orthogram. This will provide a base for the determination of the degree of shrinkage due to fixation.

When the steam formed in (*A*) has built up sufficient pressure, the clamp (*P*) is slowly opened. The side arm (*D*) is gradually closed with a rubber stopper. This must be done very carefully. By no means should the lung be allowed to collapse at

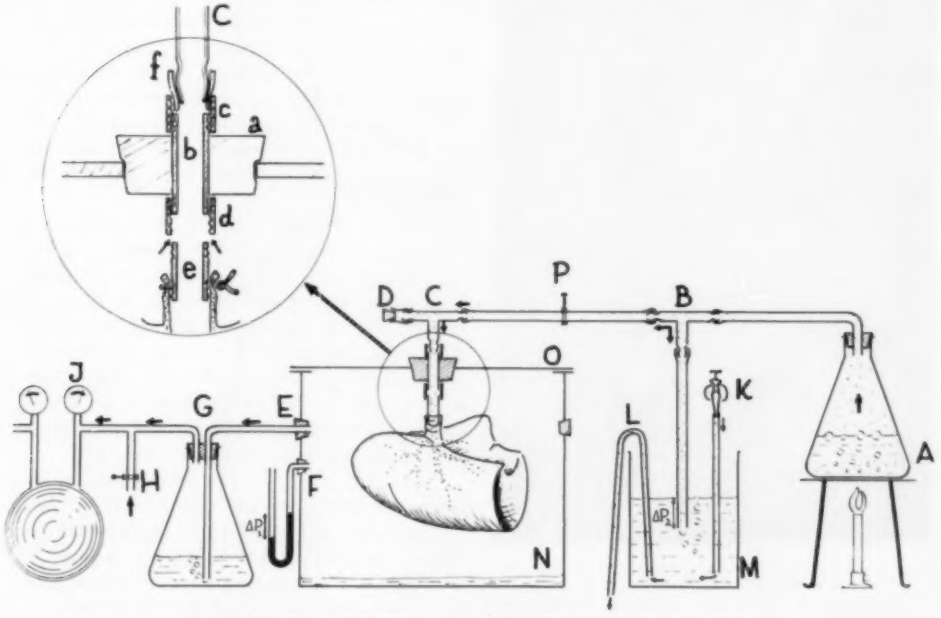

FIG. 1. Diagram of apparatus used for fixation (compare text).

this stage. In the beginning, a great amount of steam may be "consumed" by the lung, more than is produced. (*D*) has to remain partially open until the steam pressure is sufficient to maintain the lung in an inflated state. This is ensured if an excess of steam is seen bubbling out of the escape valve (*B*). The amount of steam production is controlled by varying the heat.

When an equilibrium is reached, it is maintained for one and one-half to two hours until the surface of the lung appears uniformly gray-brown. The negative pressure in the box may be reduced after some time.

At the end of the procedure, the heat at (*A*) is turned off. The clamp (*P*) is rapidly closed and the side arm (*D*) is immediately opened. Great care has to be taken that the airways should be immediately opened to air after clamping the formalin steam line. The cooling vapors inside the lung reduce their volume considerably. This would cause the lung to collapse if (*D*) remained closed; at this point the lung can no longer be re-expanded. If the external pressure never exceeds the internal pressure, however, the lung will maintain its inflated shape. The vapor cooling in (*A*) will cause water from (*M*) to flow through (*B*) into (*A*), thus diluting the strong formaldehyde solution.

When the lung has cooled off, it is disconnected from (*C*) and carefully immersed in Zenker's fluid in a large bucket; a layer of cotton, wet with fluid, is placed over the floating lung. Care has to be taken not to crush the lung in handling. It is best held by the pipe (*Ce*) which is left in place.

By the next morning, Zenker's fluid has infiltrated and hardened a shell of approximately 3 to 4 cm. in depth. The lung can now easily be handled and cut into slices of 1 to 2 cm. thickness by the aid of an 18-inch long Gillette Stady blade without collapsing (figure 2). The slices are loosely wrapped together with gauze and reimmersed in the Zenker's solution for twelve hours. The tissue of the lung then is stiff enough to withstand manipulation, slicing, embedding, et cetera, without collapsing.

FIG. 2. Median section through lung of young woman; section fixed with formalin steam. Lung was allowed to stay in Zenker solution overnight. Discolorations are due to uneven penetration of Zenker fluid.

OBSERVATIONS

Evaluation of the Method

Labor and apparatus: The method is very rapid. Within about two hours following removal of the lung, a specimen fixed in a controlled inflated state is obtained. The tissue is already sufficiently stiff to allow careful cutting of blocks. The method may, therefore, have some application when relative rapidity counts. Secondary treatment with Zenker's solution, however, yields better results. The apparatus is relatively simple and—particularly—easily set up, once its "right" and "left" portions are permanently assembled.

Quality of fixation: The rapidity of fixation is due to the application of hot concentrated formalin steam. The heat may cause some handicaps under special circumstances, as discussed below; but the general quality of fixation is good. The bronchial wall is intact; often secretions can be seen covering the cilia of the epithelium (figure 3). The architecture of the alveoli (figure 4) and the fine structure of alveolar membranes are very well preserved (figure 5).

Controlled inflation: The arrangement of the apparatus allows the lung to be inflated by negative pressure with room air or any gaseous medium to any desired degree. When a steady state of inflation is maintained, the fixative can gradually be applied to the lung through the airways.

So far there have been no means to determine accurately the degree of inflation of the lung

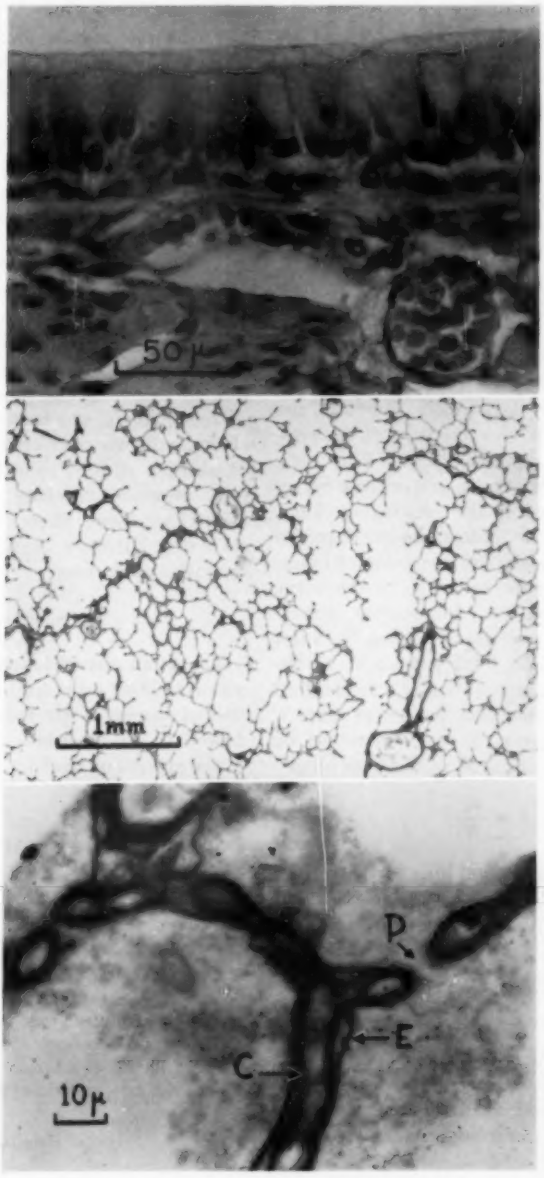

FIG. 3 (Top): Bronchial epithelium of dog's lung fixed with formalin steam. Note fine layer of mucus covering cilia. Bronchial vessels are not distorted. (Hematoxylin and eosin stain)

FIG. 4 (Center). Section of lung parenchyma from formalin-steam-fixed lung.

FIG. 5 (Bottom). Interalveolar membrane from human lung fixed with formalin steam. Note interalveolar pore *P*. Granular content of alveolus and separation of basement membranes of capillaries *C* and epithelium *E* due to pulmonary edema. Stained with periodic-acid-Schiff stain.

TABLE 1

CORRECTION FACTORS FOR ARTIFICIAL SHRINKAGE OF LUNG TISSUE DUE TO STEAM FIXATION AND PARAFFIN EMBEDDING

Conversion		Dimensions X		
From	To	Linear	Area	Volume
Fixed tissue	Fresh tissue*	1.22	1.50	1.82
Slide	Fixed tissue	1.09	1.18	1.28
Slide	Fresh tissue*	1.35	1.82	2.42

* In state of submaximal inflation (¾ "total lung capacity").

prior to fixation. The use of a plethysmograph is not reliable since these preparations can rarely be made completely leak-proof. Therefore, use has been made of the characteristics of the pressure-volume curve of the lungs (9). With some experience, the point of inflection of these curves can well be observed on the inflating specimen. An attempt was made to maintain the degree of inflation reached just above the point of inflection, making use of the hysteresis of this curve occurring in deflation (9). After fixation, the volume of the fixed lung was measured by water displacement, and the total volume V_L of the fresh lung (air + tissue + blood) was obtained by applying the conversion factors of table 1. The degree of inflation J was determined by dividing V_L by the weight W of the fresh lungs[5]:

$$J = \frac{V_L}{W}$$

If an average normal "total lung capacity" of 7 liters for the adult and an average weight of the adult lung at autopsy of 1,000 gm. are accepted, then

$$J_{TLC} \approx \frac{7000 + 1000}{1000} = 8.$$

The procedure described above resulted in a value of $J = 6.2 \pm 0.2$, so that a degree of inflation was achieved corresponding to about three-fourths "total lung capacity."

Determination of artificial shrinkage: The onset of fixation of the tissue by the warm formaldehyde will induce shrinkage, and a gradual reduction of the lung size can be observed. In addition, the lung retains some ability to recoil, which will cause a minute reduction of the lung size after the pressure gradient ceases. Drawing an orthogram of the freshly inflated lung and determination of the dimensions and volume of the fixed specimen will allow quantitative determination of this shrinkage, which in the present material reduced linear dimensions by about 18 per cent in average. From these data, correction factors for conversion of dimensions to the fresh state at about three-fourths total inflation can be derived (table 1).

Limitations of the method: If the lungs are very edematous, the fixation may sometimes be incomplete. This is due mainly to the difficulty of bringing a sufficient amount of formaldehyde into the fluid-filled parenchyma. In such cases, prolongation of the fixation time over two to three hours may overcome these difficulties. The application of heat seems to induce a particularly intensive shrinkage of dense collagenous connective tissue fibers. Extensive fibrosis or scars may, therefore, cause a moderate degree of distortion of the lung.

[5] The writers wish to thank Dr. Harry B. Martin for suggesting this relationship.

SUMMARY

The application of formalin steam through the airways allows rapid fixation of the lung in any desired degree of air inflation. Apparatus and procedure are described. The histologic quality of the specimens fixed by this procedure is very good.

SUMARIO

Fijación del Pulmón por Vapores de Formalina en un Estado Fiscalizado de Inflación con Aire

La aplicación de vapores de formalina a través de las vías aéreas permite la rápida fijación del pulmón a cualquier grado de inflación con aire que se desee. Se describen el aparato y el procedimiento. La calidad histológica de los ejemplares fijados con este método es muy buena.

RESUME

Fixation du poumon par de la vapeur de formol dans des conditions contrôlées d'inflation par air

L'usage de vapeur de formol dans les voies aériennes permet une fixation rapide du poumon à n'importe quel degré de distension par de l'air.

Les auteurs décrivent l'appareil et le procédé. La qualité histologique des échantillons fixés par ce procédé est très bonne.

Acknowledgments

The writers wish to acknowledge their sincere gratitude to Drs. Averill A. Liebow, André Cournand, and Dickinson W. Richards for their interest and support of this work, and to Mr. Frank Cappiello for his invaluable technical assistance.

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THE EFFECT OF ENDOTOXIN ON TISSUE CULTURES OF SPLEEN OF NORMAL AND TUBERCULIN-SENSITIVE ANIMALS[1, 2]

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INTRODUCTION

Cellular sensitivity of the tuberculin type has been shown to be rather specific under different experimental conditions since it was first described by Rich and Lewis (1). Tests made with various chemical fractions of *Mycobacterium tuberculosis* have indicated that only those containing protein caused specific damage to sensitive cells, the nucleic acid and polysaccharide components being without specific toxicity (2, 3). Until recently, little has been published concerning the reaction of tuberculin-sensitive cells to bacterial products other than those derived from mycobacteria. Moen (4) demonstrated the specificity of the cytotoxic reaction by showing that splenic cells of tuberculous guinea pigs were damaged *in vitro* by tuberculin but not by an extract of a *group C* hemolytic Streptococcus, whereas similar cells from guinea pigs infected with *Streptococcus pyogenes, group C*, were selectively damaged by the streptococcal antigen but not by tuberculin. Recently, Heilman and co-workers (5) have observed the same type of specificity in tissue cultures of spleen from tuberculous guinea pigs and those with experimental brucellosis. The test substances used in the experiments, tuberculin (PPD-S) and nucleoprotein from brucellae, caused cellular damage only in cultures of spleen from animals with the corresponding infection.

In recent years the specificity of the cytotoxic reaction has been questioned by Packalén and his associates (6–8). They sensitized guinea pigs to tuberculin with BCG vaccination, followed by repeated injections of heat-killed tubercle bacilli emulsified in paraffin oil. In studies on the migration of leukocytes in capillary tubes, Wasserman and Packalén (6) observed that cells from tuberculin-sensitive guinea pigs were not only more sensitive to tuberculin than normal cells, but were also somewhat more sensitive to a culture filtrate of *Escherichia coli*, and to lipopolysaccharide from *E. coli* and *Serratia marcescens*. Old Tuberculin decreased the migration of sensitive leukocytes in concentrations of 1:100 and 1:1,000 but, when effective preparations of endotoxin were diluted more than one twofold step, the inhibitory action on normal and sensitive cells was abolished. Tunçman and Packalén (7) observed similar damage to tuberculin-sensitive fibroblasts from lung and spleen in media containing tuberculin or a culture filtrate of *E. coli*. The differences were not as great as in the study on leukocytes, and it was not always possible to distinguish between normal and sensitive cells on the basis of values obtained in individual experiments, but the differences observed were statistically significant. The effect of tuberculin was greater than that of *E. coli* filtrate. Packalén, Tunçman, and Wasserman (8) reported similar results with renal epithelium growing in plasma clot cultures.

In the present experiments, no attempt was made to duplicate the methods used by Packalén and his associates. The writers were interested in observing the effect of endotoxin in a tissue culture system they have used for quantitative studies on bacterial allergy. Because of work in progress with human tissue, interest was focused primarily on the reaction of cells from tuberculin-sensitive animals showing little or no active infection. For this reason experiments were done with the spleen from guinea pigs sensitized with heat-killed mycobacteria in paraffin oil, and from rabbits infected with *M. tuberculosis* H37Rv from one to seven and one-half months previously. The comparative effect of tuberculin and endotoxin on cells from guinea pigs with experimental tuberculosis due to *M. tuberculosis* H37Rv was also observed.

MATERIALS AND METHODS

Animals: Adult male albino rabbits and adult male guinea pigs, both albino and of mixed color,

[1] From the Tissue Culture Laboratory and Medical Service, Mount Alto Veterans Administration Hospital, Washington 7, D. C.

[2] Presented in part at the annual meeting of the Eastern Section, American Thoracic Society, Baltimore, Maryland, October 28, 1960; and at the Veterans Administration Annual Research Conference, Cincinnati, Ohio, December 1, 1960.

were used. Experimental tuberculosis was produced in rabbits and guinea pigs by the administration of 1 ml. of a saline suspension containing 0.1 mg. (wet weight) of *M. tuberculosis* H37Rv. The infecting dose was given intravenously to rabbits and subcutaneously to guinea pigs. A group of guinea pigs was sensitized by a single intramuscular inoculation of sterile paraffin oil containing 5 mg. (dry weight) of heat-killed human tubercle bacilli (strain H37Rv). Animals sensitized with *M. tuberculosis* were given an intradermal test with 0.1 ml. of a 1:20 solution of Old Tuberculin. Tuberculous guinea pigs tested fifty days after infection all showed a 4+ reaction, with areas of central necrosis. Tuberculous rabbits and guinea pigs sensitized with heat-killed *M. tuberculosis* were given intradermal tests within thirty days of their use as a source of tissue. Animals selected for tissue culture experiments had characteristic delayed reactions measuring more than 12 mm. across (average diameter).

Two human spleens were also included in this study. One was removed from a 25-year-old female with traumatic rupture of the spleen (*C.L.*) and the other was from a 47-year-old man with advanced carcinoma of the stomach (*L.W.*). Both patients were positive reactors to 0.0001 mg. of PPD, but in neither case was there a history or present evidence of clinical tuberculosis.

Test substances: A 1:1,000 stock solution of PPD[3] prepared with sterile distilled water was stored at 4°C., and further dilutions were made with Tyrode's solution as needed for each experiment. The same solution of PPD was used over a period of a year, and *in vitro* assays at the beginning and a few weeks before the end of the study showed that it was as active as PPD-S. Consequently, a concentration of 5 γ per ml. of tissue culture medium was used in most of the experiments.

A filtrate of *E. coli* was prepared by Seitz filtration of an eight-day-old tryptic digest broth culture of a strain of *E. coli* recently isolated from the urine of a patient with cystitis. The filtrate was stored in ampules at 4°C., and was diluted with Tyrode's solution just before use. After culture studies were completed, the filtrate was tested and found to cause hyperthermia in the rabbit.

Lipopolysaccharide from *E. coli* (Difco lot 0127 B 8) was suspended in Tyrode's solution and heated in a water bath at 70°C. for one hour. Further dilutions of the stock solution were made with Tyrode's solution for each experiment.

Tissue culture methods: The general methods used were similar to those employed in previous studies (5). Fresh homologous serum and heparinized plasma were used in the preparation of all tissue culture media. Experimental animals were fasted for twenty-four hours prior to obtaining blood, and human donors were bled after an overnight fast. Serum-chick embryo extracts were made by extracting eight-day chick embryos with serum in the proportion of one embryo to 4 ml. of guinea pig serum or 5 ml. of rabbit or human serum. Dihydrostreptomycin was added to culture media in a concentration of 100 γ per ml. To prevent lysis of the clot, cultures of human tissue also received soy bean trypsin inhibitor in a concentration of 1:50 of an 0.5 per cent solution.

At the time cultures were made, the substances to be tested—PPD or endotoxin—were added to the serum extract to comprise one twentieth of the volume of the medium. The spleens of both a normal and a tuberculin-sensitive animal were used in comparative experiments whenever possible. Care was taken to select as controls rabbits or guinea pigs of approximately the same age and size as the sensitized animal in each experiment. Fragments of each spleen were prepared, and those measuring approximately 1.5 to 2 mm. in diameter were matched for size and placed in two to four groups of twelve fragments each. One group of explants was used in control cultures, and the remaining groups comprised the test series to which different concentrations of endotoxin or tuberculin were added.

Cultures were placed in specially manufactured D5 Carrel flasks, and consisted of 0.5 ml. of plasma, 1.0 ml. of tissue extract, and four explants of spleen placed in the medium before clotting occurred. Cultures were incubated at 37°C. in a rapid-circulation type of incubator.

Quantitative determinations: The methods used to determine the extent of cellular migration or growth have been described previously (5). The migration of leukocytes and macrophages was determined at twenty-four and ninety-six hours of incubation, respectively. Cultures were coded and read as "unknowns." The average radius of the migration zone was determined for each explant with the use of an ocular micrometer at 60 × magnification, and the average value was estimated for each group of cultures. The toxicity of endotoxin or tuberculin was determined by comparing the values for test cultures with those observed in control cultures. The results were expressed in terms of a cytotoxic index in which a value of less than 0.90 usually indicated toxicity. The formula for calculating the cytotoxic index is given in tables 1–5. The growth of fibroblasts was determined in two experiments by projectoscopic methods on the tenth day of incubation, and the results were expressed in terms of the radius of the growth zone. The growth of fibroblasts is more variable than that of the wandering cells. Statistical analysis of the data was carried out as described previously (5).

The pathologic findings in rabbits and guinea pigs infected with the human strain of *M. tuberculosis* were characteristic for each species and will not be given here. A description of the histologic changes occurring in cultures at different periods of incubation will be the subject of a separate report.

[3] The purified protein derivative of tuberculin used *in vitro* was obtained through the kindness of Dr. E. S. Barclay of Merck Sharp & Dohme.

Observations

The effect of coli filtrate and tuberculin (PPD) on normal and tuberculin-sensitive cells was investigated first. Preliminary tests indicated that coli filtrate in a concentration of 1:100 was moderately toxic for splenic macrophages from normal guinea pigs and rabbits and that half that amount usually caused little or no decrease in cellular migration; consequently, these concentrations were used in comparative tests. Sterile broth alone in a concentration of 1:100 had no effect on the growth or migration of splenic cells from normal or tuberculin-sensitive animals.

The results of three experiments with tuberculous rabbits and three with guinea pigs sensitized with heat-killed tubercle bacilli are summarized in table 1. Some individual animal variation was noted in the response of small and large wandering cells to the toxic action of coli filtrate. The differences observed between normal and tuberculin-sensitive cells were not great except in the last two experiments, in which tuberculin-sensitive macrophages were more sensitive than normal cells to coli filtrate in one experiment, and were less sensitive in the other. Significant influences on migration were accompanied by correspondingly severe morphologic damage, which appeared to be of the same order for normal and tuberculin-sensitive cells. The concentration of tuberculin used in these experiments did not affect the migration or growth of normal cells but had a selective toxic action on tuberculin-sensitive cells, the morphologic changes being similar to those previously observed in cultures of sensitive cells containing Old Tuberculin (9).

The next series of experiments was concerned with the effect of a partially purified endotoxin from *E. coli* (Difco lot 0127BS) on splenic cells of so-called normal guinea pigs and rabbits. The results from similar studies with two human spleens are included for comparison, although they came from tuberculin-sensitive individuals. The response of splenic leukocytes to different

TABLE 1

Toxicity of a Culture Filtrate of E. Coli and of Tuberculin (PPD) for Splenic Cells of Normal and Tuberculin-sensitive Animals

Values Represent Cytotoxic Index*

Source of Tissue	Duration of Sensitization	Culture Filtrate of *E. Coli*				Tuberculin (PPD)	
		1-100		1-200		5 γ per ml.	
		Leukocytes	Macrophages	Leukocytes	Macrophages	Leukocytes	Macrophages
	days						
Normal rabbit	0	*0.81*†	*0.89*			0.93	1.12
Tuberculous rabbit	231	*0.86*	*0.79*			0.91	*0.46*
Normal rabbit	0	*0.81*	*0.49*	*0.86*	*0.69*	1.01	0.94
Tuberculous rabbit	44	*0.74*	*0.58*	*0.82*	*0.64*	*0.67*	*0.32*
Normal rabbit	0	*0.89*	*0.82*	0.94	*0.86*	1.04	1.08
Tuberculous rabbit	70	*0.91*	*0.79*	0.96	0.99	*0.81*	*0.55*
Normal guinea pig	0	*0.80*	*0.82*	*0.89*	0.97	0.91	0.99
Sensitive guinea pig	26	*0.78*	*0.83*	0.89	0.99	*0.56*	*0.60*
Normal guinea pig	0	*0.84*	0.91	*0.80*	*0.85*	1.01	0.99
Sensitive guinea pig	62	*0.82*	*0.69*	0.90	*0.88*	*0.87*	*0.57*
Normal guinea pig	0	*0.81*	*0.83*	*0.86*	*0.86*	0.97	1.04
Sensitive guinea pig	65	*0.88*	1.01	*0.89*	1.03	*0.73*	*0.77*

* Cytotoxic index = $\frac{\text{Average migration cultures with endotoxin}}{\text{Average migration controls}}$.

† Values in italics show significant variation from controls at 98 per cent confidence levels.

TABLE 2

EFFECT OF ENDOTOXIN FROM E. COLI ON CULTURES OF MAMMALIAN SPLEEN

Values Represent Cytotoxic Index*

Source of Tissue	Concentration of Endotoxin in γ per Ml.									
	100	50	25	10	5	2.5	1.0	0.5	0.25	0.1
I. Toxicity of Endotoxin for Splenic Leukocytes										
Guinea pig	*0.58*†			*0.76*			*0.69*	*0.81*	*0.85*	*0.87*
Rabbit 1	*0.80*	*0.83*	*0.88*	*0.83*	*0.86*	*0.90*				
Rabbit 2						0.95	0.99	0.98	0.98	0.92
Rabbit 3				*0.71*						
Rabbit 4	*1.30*									
Human (C.L.)				*0.76*			*0.87*			1.00
Human (L.W.)				0.95			0.97			0.92
II. Toxicity of Endotoxin for Splenic Macrophages										
Guinea pig	*0.30*			*0.49*			*0.61*	*0.70*	*0.72*	*0.75*
Rabbit 1	*0.14*	*0.26*	*0.31*	*0.38*	*0.45*	*0.53*				
Rabbit 2						*0.53*	*0.63*	*0.67*	*0.85*	0.90
Rabbit 3				*0.47*						
Rabbit 4	*0.37*									
Human (C.L.)				*0.39*			*0.69*			0.91
Human (L.W.)				*0.40*			*0.74*			0.98

* Cytotoxic index $= \frac{\text{Average migration cultures with endotoxin}}{\text{Average migration controls}}$.

† Values in italics show significant variation from controls.

concentrations of endotoxin was variable (table 2). This was particularly true in cultures containing 100 γ per ml. of endotoxin, in which values were obtained that were 42 per cent less and 20 per cent greater than normal. At twenty-four hours of incubation, the outer edge of the migration zone consisted chiefly of granulocytes. Microscopic examination of unstained cultures showed these cells to be somewhat more sensitive to endotoxin than lymphocytes, judging by the distance they migrated and their relative numbers in different parts of the migration zone. Tuberculin-sensitive granulocytes were also somewhat more sensitive to tuberculin than lymphocytes, as previously observed (9).

The action of endotoxin on splenic macrophages was surprisingly regular, i.e., increasing the concentration of endotoxin caused a progressive decrease in extent of migration and correspondingly severe morphologic changes. Cellular damage due to endotoxin appeared greater than that produced by tuberculin in susceptible cultures. A detailed study of these changes will be presented elsewhere. The degree of toxicity due to endotoxin was about the same for the three animal species. The writers decided to use 1 γ per ml. of endotoxin in comparative tests, and also included a lower concentration than this, either 0.1 or 0.05 γ per ml.

Experiments with spleen from guinea pigs sensitized with heat-killed tubercle bacilli in mineral oil are presented in table 3. Endotoxin had a somewhat greater toxic effect on tuberculin-sensitive leukocytes than on normal cells. The effect on macrophages, although subject to individual variation, was about the same in the two groups. Tuberculin did not influence the migration of normal cells but specifically damaged sensitive wandering cells, especially the macrophage. It should be mentioned that all three of the sensitized guinea pigs had extensive draining abscesses at the site of injection of the tubercle bacilli in mineral oil. Bacteriologic culture of the lymph nodes draining the area showed gram-positive cocci and gram-positive and gram-negative bacilli to be present.

In the next group of experiments, endotoxin was equally toxic for wandering cells of normal

TABLE 3

EFFECT OF ENDOTOXIN FROM E. COLI AND OF TUBERCULIN (PPD) ON THE MIGRATION OF CELLS FROM EXPLANTS OF SPLEEN OF NORMAL AND TUBERCULIN-SENSITIVE GUINEA PIGS

Cytotoxic Index*

Guinea Pigs	Duration of Sensitization	*Escherichia Coli* Endotox.				Tuberculin (PPD)	
		1 γ per Ml.		0.05 γ per Ml.		5 γ per Ml.	
		Leukocytes	Macrophages	Leukocytes	Macrophages	Leukocytes	Macrophages
	days						
Normal	0	*0.79*†	*0.53*	*0.86*	*0.71*	0.98	0.98
Sensitive	117	*0.71*	*0.61*	*0.79*	*0.84*	*0.68*	*0.45*
Normal	0	*0.84*	*0.60*	*0.88*	*0.73*	0.93	0.95
Sensitive	127	*0.64*	*0.76*	*0.81*	*0.88*	*0.88*	*0.69*
Normal	0	*0.89*	*0.77*	0.92	*0.87*	1.03	1.01
Sensitive	134	*0.75*	*0.73*	*0.87*	*0.88*	*0.77*	*0.67*

* Cytotoxic index = $\frac{\text{Average migration cultures with endotoxin}}{\text{Average migration controls}}$.

† Values in italics show significant variation from controls.

TABLE 4

EFFECT OF ENDOTOXIN FROM E. COLI AND OF TUBERCULIN (PPD) ON CULTURES OF SPLEEN OF NORMAL AND TUBERCULOUS RABBITS

Cytotoxic Index*

Rabbits	Duration of Infection	*E. Coli* Endotoxin						Tuberculin (PPD)		
		1 γ per Ml.			0.1 γ per Ml.			5 γ per Ml.		
		Leukocytes	Macrophages	Fibroblasts	Leukocytes	Macrophages	Fibroblasts	Leukocytes	Macrophages	Fibroblasts
	days									
Normal	0	*0.77*†	*0.48*		*0.82*	*0.62*		1.01	0.94	
Tuberculous	44	*0.67*	*0.33*		*0.72*	*0.53*		*0.67*	*0.34*	
Normal	0	*0.79*	*0.63*	0.99	*0.90*	*0.76*	1.09	1.04	1.08	1.02
Tuberculous	70	*0.76*	*0.73*	1.13	*0.88*	*0.88*	1.03	*0.81*	*0.55*	*0.54*
Normal	0	0.96	*0.60*		1.04	*0.87*		0.97	1.03	
Tuberculous	103	1.01	*0.50*		1.05	*0.69*		0.94	*0.64*	
								10 γ per Ml.		
								Leukocytes	Macrophages	Fibroblasts
Normal	0	*0.81*	*0.88*	1.23	*0.86*	1.03	0.91	0.96	1.08	1.13
Tuberculous	110	0.92	*0.66*	0.85	*0.89*	*0.81*	0.99	*0.81*	*0.57*	*0.46*
Normal	0	*0.78*	*0.61*		*0.82*	*0.70*		0.95	1.01	
Tuberculous	126	*0.87*	*0.86*		0.92	*0.88*		*0.78*	*0.63*	

* Cytotoxic index = $\frac{\text{Average migration or growth cultures with endotoxin}}{\text{Average migration or growth controls}}$.

† Values in italics show significant variation from controls.

TABLE 5

EFFECT OF ENDOTOXIN FROM E. COLI AND OF TUBERCULIN (PPD) ON CULTURES OF SPLEEN FROM NORMAL GUINEA PIGS AND GUINEA PIGS INFECTED WITH MYCOBACTERIUM TUBERCULOSIS H37Rv

Cytotoxic Index*

Guinea Pigs	Duration of Infection	*E. Coli* Endotoxin				Tuberculin (PPD)	
		1 γ per Ml.		0.05 γ per Ml.		5 γ per Ml.	
		Leukocytes	Macrophages	Leukocytes	Macrophages	Leukocytes	Macrophages
	days						
Normal	0	*0.86*†	*0.81*	0.94	0.94	0.97	0.91
Tuberculous	54	*0.81*	*0.74*	0.97	0.91	*0.72*	*0.40*
Normal	0	0.87	*0.67*	1.07	*0.83*	1.10	0.94
Tuberculous	55	*0.76*	*0.62*	0.95	*0.84*	*0.72*	*0.36*
Normal	0	0.85	*0.70*	*1.13*	1.03	*1.22*	*1.12*
Tuberculous	57	0.91	*0.70*	1.05	1.01	*0.79*	*0.53*
Tuberculous	62	*0.78*	*0.71*	*0.88*	*0.85*	*0.68*	*0.54*
Normal	0	*0.80*	*0.72*	*0.89*	*0.89*	0.94	0.92
Tuberculous	71	*0.76*	*0.67*	*0.83*	*0.83*	*0.72*	*0.50*

* Cytotoxic index = $\frac{\text{Average migration cultures with endotoxin}}{\text{Average migration controls}}$.

† Values in italics show significant variation from controls.

or tuberculous rabbits (table 4). Macrophages were more sensitive than leukocytes in most instances. Tuberculin in concentrations of 5 or 10 γ per ml. had a specific toxic action on sensitive cells, particularly macrophages and fibroblasts, which were equally sensitive in these experiments. Fibroblastic growth was not affected by 1 γ per ml. of endotoxin.

The last group of tuberculin-sensitive animals studied consisted of guinea pigs infected with *M. tuberculosis* strain H37Rv (table 5). As might be expected, these animals showed a greater degree of cellular sensitivity to tuberculin than the other groups; however, the reaction of their splenic cells to endotoxin was substantially the same as that of normal guinea pigs. In general, the large wandering cells of animals with active infection migrated farther than those of uninfected animals. In this group of experiments, a relationship between the extent of migration in tuberculin-sensitive control cultures and the degree of sensitivity to endotoxin was not observed.

DISCUSSION

Although tuberculin-sensitive and normal cells reacted in a similar way to endotoxin, this does not necessarily contradict the findings of Packalén and his associates. The present experiments differed from theirs in several respects; namely, other methods of sensitizing animals were used, as well as a different kind of medium for growing tissues. It has often been shown that even minor changes in tissue culture methods can greatly affect the results in studies on bacterial allergy. In the present studies, the primary interest was in the reaction of the macrophage. Studies are in progress to determine the relative sensitivity of normal and tuberculin-sensitive fibroblasts to increasing amounts of endotoxin.

The fact that granulocytes and macrophages are actively phagocytic may account for the greater endotoxic damage to these cells as compared with lymphocytes and fibroblasts. This selective damage was seen not only with purified endotoxin, which is particulate, but also in experiments with coli filtrate. Although the behavior of endotoxin in solution is complex, observations of Westphal and Lüderitz (10) indicated that aggregates are present which consist of spherelike units having a particle weight of about one million. Ribi and associates (11) estimated a similar particle weight for active fractions from cultures of *Salmonella enteritidis*.

This is much greater than the particle size of active components of PPD-S, which Seibert has determined to be between 9,000 and 44,000 (12). In this connection, experiments with whole heat-killed *Br. suis* showed that the active substance responsible for the allergic reaction is diffusible and capable of causing damage to Brucella-sensitive fibroblasts as well as phagocytic cells (13). In view of the differences in physical state of the substances tested, the relative sensitivity of tuberculin-sensitive fibroblasts to the two substances cannot be taken as conclusive evidence for either a basic similarity or difference in their mode of action on the cell. In studies with mixed-cell populations, further experimentation is often necessary to decide whether the effects observed are primary or secondary. The relatively low sensitivity of the fibroblast to endotoxin may also be the result of other factors, a possible influence being partial detoxification by phagocytic cells (14).

The degree of inhibition of leukocytic migration by endotoxin in the present study was similar to that observed by Wasserman and Packalén (6), and agrees with the report of Martin and Chaudhuri (15), who used the slide-cell technique to determine the effect of various bacterial products on the migration of human leukocytes. These workers found that gram-negative bacteria or their products decreased migration, *Salmonella typhi* endotoxin being toxic in concentrations of 0.058 to 0.58 γ per ml., whereas 2,000 γ per ml. of pneumococcal polysaccharide were required to inhibit migration. They observed a marked variation between individuals in the cellular response to endotoxin.

In contrast to the irregular response of leukocytes to endotoxin is the rather regular and marked toxic effect on the macrophage. This is consistent with the transient depression of phagocytic activity of the reticuloendothelial system demonstrated by Biozzi and associates (16), and the observations of Smith and coworkers (17) that the first dose of endotoxin greatly reduces the ability of the liver to remove a second dose given twenty-four hours later. The present investigators did not observe species' differences in a limited number of experiments, although man is more sensitive to endotoxin than the guinea pig or rabbit. The amount of endotoxin causing damage *in vitro* is large compared with doses lethal for rabbits and guinea pigs. This is offset somewhat by the ability of the macrophage to take up circulating endotoxin and to engulf leukocytes injured by the ingestion of endotoxin. Damage to a certain proportion of migrating cells could result from low concentrations of endotoxin without causing a decrease in extent of migration of all of the cells in the advancing border.

That the macrophage was more sensitive to endotoxin than other splenic cells appears to be important with regard to the so-called "heteroallergic" focal reaction. A general stimulation of the reticuloendothelial system in tuberculosis and the presence of numerous histiocytic cells in a tuberculous focus provide a means for rapid removal of endotoxin from the circulation and concentration of the toxic material in the lesion. Bordet (18) showed that guinea pigs given BCG were many times more susceptible than normal animals to the parenteral administration of culture filtrates of gram-negative bacteria. Freund (19) observed that tuberculous animals dying a few hours after injection of endotoxin showed congestion of the lungs with hemorrhagic areas around the tubercles, hemorrhagic congestion of the spleen (and sometimes of the liver and lymph nodes), and bloody exudate in the peritoneal and pleural cavities. The findings were similar to those of generalized tuberculin shock. It should be mentioned that similar focal reactions to endotoxin have been observed in lesions due to *Bacillus anthracis*, vaccinia (20), *Pasteurella pseudotuberculosis*, and talc granulomas (18). Bordet (21) followed the course of the reaction in guinea pigs given BCG and in those with talc granulomas, and concluded that the presence of inflammatory tissue reactions was necessary for an augmented sensitivity to endotoxin. Animals regained their normal reactivity to endotoxin as the inflammatory tissue reactions subsided. These early observations are of special interest in view of recent studies on the role of the reticuloendothelial system in endotoxic shock. Howard and his associates (22), in experiments with mice previously infected with BCG, observed that the rate of carbon clearance from the circulation was increased five times, but the same animals were one hundred times more susceptible to killing by bacterial endotoxin than normal mice. The rate of breakdown of isotopically labeled endotoxin was markedly increased in mice infected with BCG, and they were more resistant than normal mice to infection with *S. enteritidis*. When stimulation of the reticuloendo-

thelial system results from repeated administration of sublethal doses of endotoxin, increasing phagocytic activity of the reticuloendothelial system is associated with tolerance to lethal doses of endotoxin. Whether or not tolerance is reflected at the cellular level remains to be determined. What part damage to phagocytic cells, particularly the macrophage, might play in development of intravascular fibrinoid formation and the hemorrhagic focal reaction is not known, but this observation along with other factors influencing the reaction suggest possibilities for future investigation.

In view of the variation in cellular response to endotoxin of different animals in the present writers' studies, it is possible that a large number of experiments might have revealed a statistical difference between the cells of groups of normal and tuberculin-sensitive animals. In studies reported by Packalén and his associates the most definite results were obtained with leukocytes (6), but the significance of antigenic damage to the leukocytes is not well understood, and it is not certain to what extent the damage observed is related to allergic reactions of the delayed type. The results reported by Packalén and his associates in studies with fibroblasts and renal epithelium were not as definite as those with leukocytes, and there was a good deal of overlapping of the data obtained with normal and tuberculin-sensitive animals (7, 8). It seems unlikely that these differences observed at a cellular level could account for a great increase in sensitivity of tuberculous animals to endotoxin. The present results and those of Packalén and co-workers show that one cannot choose a concentration of endotoxin that is harmless to normal cells but uniformly toxic for tuberculin-sensitive cells. This degree of selective damage can be demonstrated with different concentrations of tuberculin (PPD) by the use of methods described in the present study.

The results of the present study support the view that the heteroallergic reaction, although related in some way to the reactivity of the reticuloendothelial system, is independent of hypersensitivity to tuberculin. In this connection, Suter and associates (23) showed that the susceptibility of mice to endotoxin could be enhanced not only by BCG, but also to a smaller degree by previous administration of cord factor which did not cause the development of hypersensitivity. Hall and Atkins (24) have shown that tuberculin fever is due to an endogenous pyrogen released by the specific action of tuberculin on sensitive cells. Cross-tolerance between endotoxin and tuberculin could not be demonstrated. Stetson (25) has discussed certain similarities between the local reactions to endotoxin and those of delayed hypersensitivity. The reaction of the macrophage *in vitro* may be a useful tool for studying these relationships as well as certain facets of the Shwartzman reaction and endotoxic shock.

Summary

The relative toxic effect of tuberculin (PPD) and of *E. coli* filtrate or endotoxin for splenic cells of normal and tuberculin-sensitive animals was determined in tissue culture. The degree of toxicity was estimated by quantitative determinations of the relative effect on migration or growth of wandering cells and fibroblasts from explants of spleen maintained in a semisolid tissue culture medium.

The concentration of PPD employed did not affect normal cells but definitely decreased the migration and growth of leukocytes, macrophages, and fibroblasts from tuberculin-sensitive animals.

Escherichia coli endotoxin in concentrations between 0.25 and 100 γ per ml. of culture medium had a variable effect on leukocytes, but a fairly regular and marked toxic effect on macrophages from man, rabbits, and guinea pigs. The relative sensitivity of the macrophage to endotoxin was similar in the three species.

Although some variation existed in the cellular sensitivity of individual animals, splenic leukocytes and macrophages from tuberculin-sensitive rabbits and guinea pigs were not found to be more sensitive to coli filtrate or endotoxin than the corresponding cells from normal animals. The results confirm the specificity of the method for studying delayed allergy.

The highest concentration of endotoxin used in comparative tests (1 γ per ml.) did not decrease the growth of normal or tuberculin-sensitive fibroblasts.

Sumario

El Efecto de la Endotoxina sobre los Histocultivos de Bazo de Animales Normales y Tuberculinosensibles

En cultivos histológicos se determinó el relativo efecto tóxico ejercido por la tuberculina (DPP)

y por el filtrado o la endotoxina de la *E. coli* sobre las células esplénicas de animales normales y tuberculinosensibles. Se calculó el índice de toxicidad por determinaciones cuantitativas del efecto relativo sobre la migración o proliferación de células errantes o fibroblastos procedentes de explantes de bazo mantenidos en un medio semisólido de histocultivo.

La concentración del DPP empleado no afectó las células normales, pero sí hizo bajar indudablemente la migración y proliferación de los leucocitos, macrófagos y fibroblastos derivados de los animales tuberculinosensibles.

La endotoxina de la *Escherichia coli* a concentraciones entre 0.25 y 100 γ por ml. de medio de cultivo ejerció efecto variable sobre los leucocitos, pero efecto tóxico bastante regular y acentuado sobre los macrófagos procedentes de seres humanos, conejos y cobayos. La sensibilidad relativa del macrófago a la endotoxina fué similar en las tres especies.

Aunque existía alguna variación en la sensibilidad celular de animales dados, los leucocitos y macrófagos esplénicos derivados de conejos y cobayos tuberculinosensibles no resultaron ser más sensibles al filtrado o la endotoxina coli que las células correspondientes procedentes de animales normales. Los resultados confirman la especificidad de la técnica para estudiar la alergia demorada.

La concentración máxima (1 γ por ml.) de endotoxina usada en las pruebas comparativas no atenuó la proliferación de los fibroblastos normales o tuberculinosensibles.

Resume

L'effet de l'endotoxine sur des cultures de tissu de rate d'animaux normaux et d'animaux sensibilisés à la tuberculine

On étudia dans des cultures de tissu l'effet relativement toxique de la tuberculine (PPD) et d'un filtrat de coli ou d'endotoxine sur des cellules spléniques d'animaux normaux et d'animaux sensibilisés à la tubercuiine. On estima que le degré de toxicité par des déterminations quantitatives de l'effet relatif sur la migration ou le développement des cellules libres et des fibroblastes de fragments de rate prélevés maintenus dans un milieu de culture de tissu semisolide.

La concentration de PPD employée n'affecta pas les cellules normales mais diminua nettement la migration et le développement des leucocytes, des macrophages et des fibroblastes des animaux sensibilisés à la tuberculine.

L'endotoxine d'*Escherichia coli* à des concentrations de 0,25 à 100 γ par ml de milieu de culture eut un effet variable sur les leucocytes, mais un effet assez régulier et nettement toxique sur les macrophages de l'homme, des lapins et des cobayes. La sensibilité relative des macrophages à l'endotoxine fut semblable dans les trois espèces.

Bien qu'il existe quelques variations dans la sensibilité cellulaire d'animaux, individuellement, les leucocytes spléniques et les macrophages de lapins et de cobayes sensibilisés à la tuberculine ne furent pas, trouva-t-on, plus sensibles au filtra de coli ou à l'endotoxine que les mêmes cellules d'animaux normaux. Les résultats confirment la spécificité de la méthode dans l'étude de l'allergie retardée. La plus forte concentration d'endotoxine utilisée dans des tests comparés (1 γ par ml) ne diminua pas le développement des fibroblastes normaux ou sensibles à la tuberculine.

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THE EFFECT OF 5-BROMOSALICYLHYDROXAMIC ACID ON THE ACETYLATION OF ISONIAZID AND ITS CONCENTRATION IN THE BLOOD[1]

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Introduction

In a number of experimental and clinical studies in laboratory animals and man, it has been reported that the administration of para-aminosalicylic acid (PAS) and other aromatic amines in combination with isoniazid gives rise to elevated and prolonged concentrations of free isoniazid (1–8), due presumably to competitive inhibition of isoniazid acetylation (1, 9, 10). However, it should be noted that the results of one study have been interpreted as suggesting that PAS exerts no significant influence on the metabolism of isoniazid in man (11), although this interpretation has been questioned by others (12). It has also been reported that the quantity of free isoniazid in the blood, as influenced by isoniazid dosage level and rate of metabolic inactivation, appears to be related in a direct manner to the emergence of bacterial resistance to isoniazid therapy in tuberculous patients (5, 13–15). These studies have been interpreted as showing that high doses of isoniazid and low rate of inactivation are associated with a low incidence of isoniazid resistance. It is conceivable, therefore, that the ability of PAS to delay the emergence of resistance to isoniazid may be due in large part to its effect on the concentration of isoniazid in the blood, in addition to the antibacterial activity of PAS independent of isoniazid (16).

Hornung and associates (17) recently reported that 5-bromosalicylhydroxamic acid (BSH), 3 gm. daily, was as effective as PAS, 10 gm. daily, in delaying the emergence of resistance to isoniazid in tuberculous patients. In view of the extremely low *in vitro* antibacterial activity of BSH, 62.5 γ per ml. being required to inhibit the growth of *Mycobacterium tuberculosis* H37Rv in Dubos medium (18), the possibility was considered that BSH may exert its effect predominantly by inhibiting the inactivation of isoniazid. Consequently, BSH was tested for its inhibitory effect on isoniazid acetylation *in vitro*, and its ability to increase the concentrations of free isoniazid in the blood of rabbits was ascertained. On the basis of the results obtained in these experiments, studies were carried out in tuberculous patients to compare the effect of BSH with that of PAS on serum concentrations of antimicrobially active isoniazid.

Materials and Methods

The methods used for the *in vitro* acetylation experiments and the *in vivo* studies with rabbits were those described previously (1), except that the rabbits used in the present experiments were a cross between the white albino and the California rabbit, each weighing 2 to 3 kg.

Sixty-four tuberculous patients at the Royal Edward Laurentian Hospital were selected as subjects for investigation of the comparative effect of BSH and PAS on serum concentrations of isoniazid. They were divided at random into two groups of 32 patients each. The patients in both groups received their usual therapeutic doses of isoniazid, 100 to 125 mg. three times daily, but no other drug, for three days prior to the test. The test dose of isoniazid (150 mg.) was administered at 8 a.m. of the fourth day. For the next three days, *group 1* received 4 gm. of PAS and *group 2* received 1 gm. of BSH three times daily, along with 100 to 125 mg. of isoniazid. At 8 a.m. of the fourth day, a test dose of 150 mg. of isoniazid plus 4 gm. of PAS was administered to *group 1*, and 150 mg. of isoniazid plus 1 gm. of BSH were given to *group 2*. All drugs were administered in tablet form. Three hours following the administration of each test dose, blood specimens were drawn for isoniazid assay. Serum isoniazid concentrations were determined by the microbiologic method of Mandel and associates (2), except that the standard controls for each series contained 0, 0.025, 0.05, and 0.1 γ of isoniazid per ml.

Results

In vitro: The effect of the hydroxamic acids of 5-bromosalicylic acid, PAS, and salicylic acid on isoniazid acetylation by pigeon liver extracts is shown in table 1. The effects of the correspond-

[1] From the Research Laboratories, Frank W. Horner Limited, and the Royal Edward Laurentian Hospital, Montreal, Canada.

TABLE 1

INHIBITION OF ISONIAZID ACETYLATION IN PIGEON LIVER EXTRACTS BY HYDROXAMIC ACIDS AND AMIDES OF SALICYLIC ACID*

Inhibitor	Concentration of Inhibitor M/L. × 10^{-4}	Inhibition
		per cent
5-Bromosalicylhydroxamic acid (BSH)	1.0	49
5-Bromosalicylamide	1.3	52
Para-aminosalicylhydroxamic acid	2.5	65
Para-aminosalicylamide	10.0	86
Para-aminosalicylic acid (PAS)	10.0	49
Salicylhydroxamic acid	10.0	37
Salicylamide	10.0	36

* Experimental conditions as described previously (1).

ing amides and PAS, previously reported (1), are also included for purposes of comparison. The hydroxamic acids inhibited acetylation to the same extent as the corresponding amides, with the exception of the hydroxamic acid of PAS, which appears to be considerably more active than PAS amide and four times as active as PAS itself. It should be noted that the inhibitory potency of BSH is ten times that of PAS. As a matter of interest, α-ethylthioisonicotinamide, which shows marked antituberculous activity, and has been used therapeutically in combination with isoniazid (19), was tested for its inhibitory effect on isoniazid acetylation. At a 10^{-3} M concentration the inhibition was 53 per cent, which is equivalent to that obtained with PAS at the same concentration.

In vivo: 5-Bromosalicylhydroxamic acid (500 mg. per kg. of body weight) administered concurrently with isoniazid (50 mg. per kg.) to rabbits produced a marked increase in free isoniazid serum concentrations (table 2). At the four-hour interval following drug administration, the mean plasma concentration of isoniazid in the presence of BSH was 165 ± 20 per cent of that attained when isoniazid alone was given.

The results presented in table 3 show the serum concentrations of antimicrobially active isoniazid attained by tuberculous patients as measured three hours after test doses of: 150 mg. of isoniazid, 150 mg. of isoniazid plus 4 gm. of PAS, and 150 mg. of isoniazid plus 1 gm. of BSH. It can be seen that BSH at one-fourth the dosage level of PAS compared favorably with the latter as far as their effects on serum isoniazid concentrations are concerned. These results are in agreement with those of Hornung and co-workers (17) with regard to the relative effectiveness of BSH and PAS in delaying the emergence of resistance to isoniazid.

DISCUSSION

To inhibit the growth of *M. tuberculosis* H37Rv in Dubos medium, a concentration of BSH of 62.5 γ per ml. was required, as compared with 125 to 250 γ per ml. for 5-bromosalicylamide (18). As BSH is largely converted into 5-bromosalicylamide in man and the rabbit, it would be expected that the *in vivo* antituberculous activity of BSH would be even lower than that found *in vitro*, unless the glucuronides of these substances, in which form they are excreted (18), have greater antibacterial activity than the parent compounds. The present results indicate that BSH is an effective inhibitor of isoniazid inactivation *in vivo*, and it may well be that this effect is greatly responsible for its ability to delay the emergence of bacterial resistance to isoniazid, as observed by Hornung and associates (17). It would seem that BSH warrants further consideration as a potential substitute for PAS in tuberculosis therapy, particularly in the case of patients who are unable to tolerate PAS.

TABLE 2

EFFECT OF 5-BROMOSALICYLHYDROXAMIC ACID (BSH) ON PLASMA CONCENTRATIONS OF FREE ISONIAZID IN RABBITS

Hours after Dosage*	Plasma Concentrations of Free Isoniazid†		
	Without BSH	With BSH	Per Cent of Control
	γ per ml.	*γ per ml.*	
1	10.8 ± 0.7	15.2 ± 1.9	147 ± 24
2	8.4 ± 0.7	12.9 ± 0.5	159 ± 14
4	5.4 ± 0.3	8.9 ± 1.2	165 ± 20

* Isoniazid, 50 mg. per kg. of body weight administered orally with or without BSH, 500 mg. per kg.

† Mean ± standard error of 7 rabbits.

TABLE 3

EFFECT OF BSH AND PAS ON SERUM ISONIAZID CONCENTRATIONS IN TUBERCULOUS PATIENTS*

Group 1 Patients	Serum Isoniazid Concentration		Group 2 Patients	Serum Isoniazid Concentration	
	Isoniazid Alone†	Isoniazid + PAS		Isoniazid Alone	Isoniazid + BSH
	γ per ml.			γ per ml.	
1	0.5	1.0	1	1.0	4.0
2	2.0	2.0	2	0.5	2.0
3	1.0	2.0	3	1.0	2.0
4	0.5	1.0	4	1.0	2.0
5	2.0	2.0	5	0.5	1.0
6	1.0	1.0	6	1.0	2.0
7	0.25	0.5	7	2.0	4.0
8	1.0	1.0	8	0.5	1.0
9	0.5	1.0	9	0.5	1.0
10	0.25	0.5	10	1.0	2.0
11	1.0	2.0	11	1.0	2.0
12	1.0	2.0	12	0.5	2.0
13	0.5	1.0	13	2.0	4.0
14	1.0	1.0	14	1.0	2.0
15	1.0	2.0	15	1.0	4.0
16	0.5	1.0	16	1.0	2.0
17	0.5	1.0	17	0.5	1.0
18	1.0	2.0	18	0.5	1.0
19	2.0	2.0	19	0.5	1.0
20	1.0	4.0	20	2.0	4.0
21	0.5	1.0	21	0.5	2.0
22	1.0	2.0	22	1.0	2.0
23	2.0	4.0	23	0.5	1.0
24	1.0	4.0	24	1.0	4.0
25	0.5	2.0	25	2.0	4.0
26	1.0	4.0	26	0.5	1.0
27	0.5	1.0	27	1.0	2.0
28	1.0	2.0	28	1.0	4.0
29	1.0	2.0	29	0.5	2.0
30	2.0	4.0	30	1.0	2.0
31	0.5	1.0	31	0.25	1.0
32	1.0	2.0	32	1.0	2.0
Average	0.9	1.8		0.9	2.2

* Experimental conditions are described under MATERIALS AND METHODS.

† Test doses: isoniazid, 150 mg.; PAS, 4 gm.; BSH, 1 gm.

SUMMARY

The acetylation of isoniazid by pigeon liver extracts was found to be inhibited by salicyl-, 5-bromosalicyl-, and para-aminosalicyl-, hydroxamic acids. In this respect, 5-bromosalicylhydroxamic acid was ten times as potent as PAS. When given to rabbits conjointly with isoniazid, 5-bromosalicylhydroxamic acid markedly increased plasma concentrations of free isoniazid. In tuberculous patients, 1 gm. of 5-bromosalicylhydroxamic acid gave rise to the same increase in the serum concentration of antimicrobially active isoniazid as did 4 gm. of PAS.

SUMARIO

El Efecto del Acido 5-Bromosalicilhidroxámico sobre la Acetilación y las Concentraciones Séricas de la Isoniacida

La acetilación de la isoniacida por extractos de hígado de paloma quedó, según se observó, inhibida por los ácidos hidroxámicos salicílico, 5-bromosalicílico y para-aminosalicílico. En este sentido, el ácido 5-bromosalicilhidroxámico resultó diez veces más potente que el PAS. Administrado a los conejos conjuntamente con isoniacida, el ácido 5-bromosalicilhidroxámico acrecentó notablemente las concentraciones plasmáticas de isoniacida libre. En los tuberculosos, 1 gm. de ácido 5-bromosalicilhidroxámico produjo el mismo aumento que 4 gm. de PAS en la concentración sérica de isoniacida activa microbianamente.

RESUME

L'effet de l'acide 5-bromosalicylhydroxamique sur l'acétylation de l'isoniazide et les concentrations de l'isoniazide sérique

On trouva que l'acétylation de l'isoniazide par des extraits de foie de pigeon est inhibée par les acides salicyl-, 5-bromosalicyl-, et para-aminosalicyl-, hydroxamique. A ce point de vue, l'acide 5-bromosalicylhydroxamique fut dix fois plus puissant que le PAS. Quand on en donna à des lapins en même temps que de l'isoniazide, l'acide 5-bromosalicylhydroxamique augmenta nettement les concentrations plasmatiques d'isoniazide libre. Chez des malades tuberculeux, 1 gm d'acide 5-bromosalicylhydroxamique provoqua la même augmentation des concentrations sériques d'isoniazide antimicrobiologiquement actif que fut obtenue avec 4 gm de PAS.

Acknowledgment

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Case Reports

PULMONARY ALVEOLAR PROTEINOSIS[1]

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(Received for publication April 24, 1961)

INTRODUCTION

The entity of pulmonary alveolar proteinosis was first described by Rosen and associates (1) in 1958. This disease is characterized anatomically by filling of the alveoli with peculiar muco- and lipoproteinaceous material, and clinically by progressive loss of pulmonary function. In this report a case is discussed and the findings in the sputum are correlated with those of the pulmonary biopsy. It is believed that the disease may be tentatively diagnosed by examination of the sputum, as indicated by Carlson and Mason (2).

CASE REPORT

The patient, W.M.T. (Duke history number F 25492), was a thirty-one-year old Negro housewife who was referred to Duke Medical Center by the Eastern North Carolina Sanatorium on June 20, 1960. Because of recent contact with her father who had active pulmonary tuberculosis, she was given a routine examination by the county health department. A chest film revealed changes suspicious of tuberculosis, and a two-plus tuberculin test was reported. Because of these factors she was admitted to the Eastern North Carolina Sanatorium, but denied any marked respiratory symptoms. She gave a history of generalized malaise and fatigue of one year's duration. Past history revealed that she was brought up on a farm and had done housework for the past seven years. Prior to that she had worked in the tobacco fields and in a dry cleaning establishment for two years. She has three children who are living and normal. She had no serious illness except for one episode of expectoration of blood which was thought to originate from the throat.

The physical examination at that time was normal. The sputum and gastric washings were negative for acid-fast bacilli and fungi. A scalene node biopsy revealed reticuloendothelial hyperplasia. All hematologic studies were essentially normal, including a negative lupus erythematosus preparation and a serum protein of 6.4 gm. per 100 ml. Because of the strong possibility of tuberculosis, the patient was started on isoniazid and para-aminosalicylic acid therapy. Since no roentgenographic improvement of the chest resulted from this drug therapy, she was transferred to Duke Medical Center for further studies.

Physical examination on admission to Duke revealed a well developed but undernourished Negro female. She had a pulse of 88 per minute, regular; blood pressure, 100 systolic, 70 diastolic in mm. of mercury; respirations, 20 per minute; and a temperature of 98.9°F. Other physical findings were essentially normal except for the presence of wheezes over the right lung field. Laboratory data showed a hemoglobin of 14.2 gm. per 100 ml., a positive sickle cell preparation, and a leukocyte count of 4,750 with normal differentiation. Pulmonary function studies showed a maximal breathing capacity of 57 per cent and a vital capacity of 40.5 per cent, with a seven-minute helium washout, which was considered slightly prolonged. It was believed that the findings were compatible with a restrictive type of lung disease. An electrocardiogram and a serum electrophoresis were normal. Roentgenograms of the chest revealed some infiltration radiating from the right hilum extending superiorly and inferiorly. In addition, there were some patchy infiltrations in both upper and lower fields (figures 1 and 2).

Bronchoscopic examination was unrevealing, and the bronchial aspirates were negative for tumor cells, acid-fast bacilli, and fungi. Cultures of the sputum grew normal flora.

An exploratory thoracotomy revealed multiple areas of nodular infiltration in the lung. The cut surface oozed frothy pink material. There was no pleural adhesion, and inflammatory exudates were not noted. A lung biopsy was taken.

Examination of the surgically excised lung tissue revealed multiple areas of brown-gray consolidation 6 to 8 mm. in size. The pleura was normal. Microscopically (figures 3 and 4) the consolidated lesions were in a confluent lobular pattern. On the hematoxylin and eosin sections the alveoli were greatly distended and filled with an eosinophilic granular and flocculer material. In addition there were a few foamy macrophages, desquamated alveolar cells, small laminated bodies of 3–4 μ in size, disintegrating erythrocytes, acicular spaces, and occasional giant cells. In most places the alveolar septa were delicate and ischemic, but were frequently disrupted, possibly due to overdistention of the alveoli. Focal fibrosis, lymphocytic infiltration, and hypertrophy of the alveolar epithelial cells were seen. The reticulum and elastic fibers of both the pulmonary tissue and the blood vessels were not remarkable. The small bronchi were

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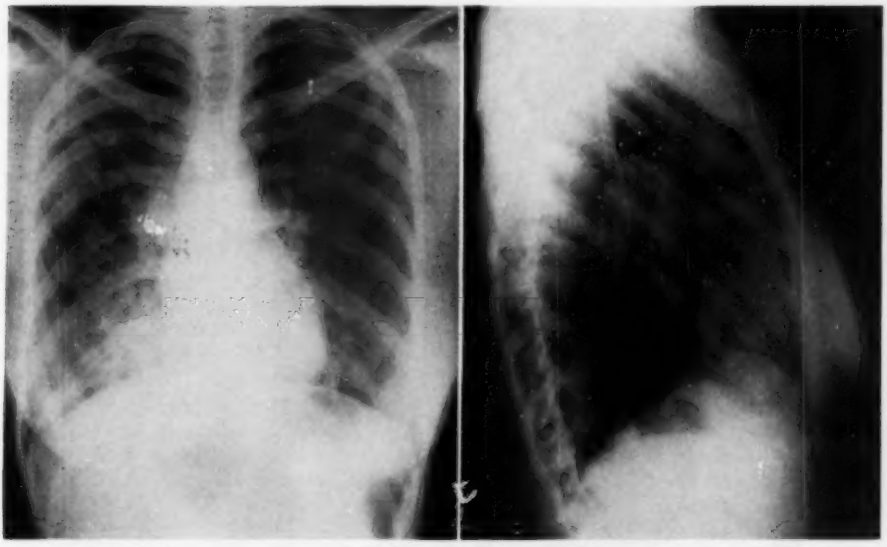

FIG. 1 (Left). Posteroanterior chest film showing diffuse "fluffy" infiltration of the right lung concentrated in the right perihilar and right lower lobe areas.

FIG. 2 (Right). Right lateral chest film showing infiltrative lesions in the posterior lower and upper lung fields.

rather contracted, and the bronchial muscles were slightly hypertrophic. The distal bronchi also contained a small amount of proteinaceous material. The bronchial epithelium was normal.

The alveolar content showed characteristic histochemical reactions, as have been indicated by Rosen and associates (1). It was positive with periodic-acid-Schiff reaction, green with the Masson trichrome stain, and orange-yellow with phosphotungstic acid hematoxylin stain. The oil red O stain on the frozen sectioned tissue revealed a small amount of fat in the macrophages and in the alveolar exudate. Many birefringent needle-shaped crystals were seen under the polarized light. The floccular material in the alveolar exudate reacted positively to luxol-fast blue stain, indicating the presence of phospholipids; however, the combined luxol-fast blue and periodic-acid-Schiff stains revealed a greater amount of mucopolysaccharides than lipids. No acid mucopolysaccharides were demonstrated with alcian blue stain. Many sections were stained with MacCallum-Goodpasture bacterial stain, acid-fast stain, Giemsa stain, acridine orange fluorescent stain for fungi, Grocott-Gomori methenamine silver stain, and Dieterle stain in a search for organisms. None could be demonstrated by any method. The cultures of the lung tissue were also negative.

Postoperatively the patient recovered uneventfully and was returned to the sanatorium. She was continued on isoniazid therapy with little change in her pulmonary condition. She was readmitted to Duke Medical Center on August 1, 1960, for follow-up evaluation. The pulmonary function tests showed a vital capacity of 61 per cent and a maximal breathing capacity of 80 per cent of expected normal value. The ratio of the residual volume to the total lung capacity was 16 per cent, and the helium washout was four minutes. Serum electrophoresis showed a slight decrease in alpha-2 and beta globulins. Her chest roentgenogram and overall physical condition remained unchanged.

Sputum samples fixed immediately in 10 per cent formalin were collected for three successive days. The specimen was centrifuged and a paraffin block was made of the solid elements. Sections were stained with hematoxylin and eosin and periodic-acid-Schiff stains. The sections showed small amounts of eosinophilic granular and floccular masses in which acicular spaces were present. The morphology and the histochemical reactions of these masses of material were identical with those found in the alveoli of the biopsied lung tissue. Giant cells and laminated bodies, however, were not found. On the other hand, a great number of gram-positive cocci and epithelial cells from the oral cavity were present.

DISCUSSION

The clinical, roentgenographic, and pathologic features presented in this case are characteristic of alveolar proteinosis. No history of inhalation of respiratory irritants, previous pulmonary dis-

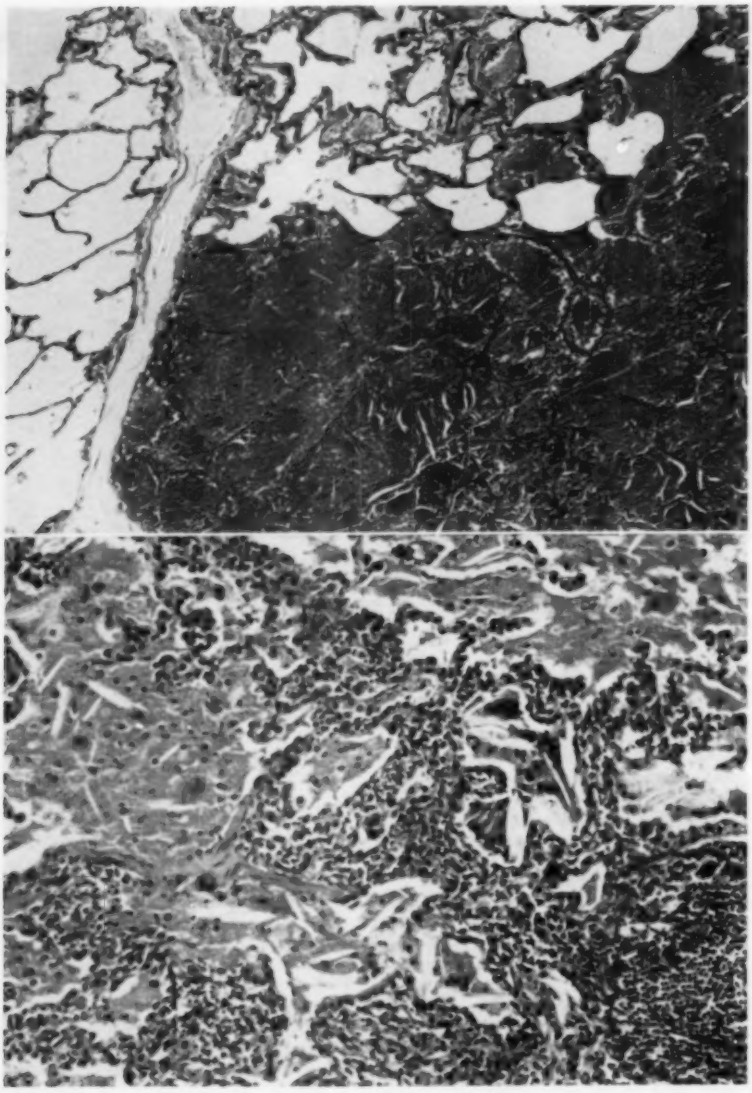

FIG. 3 (Upper). Microscopic features showing lobular consolidation of the lung with periodic acid Schiff positive material in the alveoli. The alveolar septi are thin, delicate, and lack inflammatory reaction. Many acicular spaces are present in the exudate. The adjacent pulmonary lobule appears normal. (Periodic-acid-Schiff stain, 107 magnification)

FIG. 4 (Lower). Note the lymphocytic infiltration of the alveolar septi and giant cell reaction around the acicular spaces. The alveolar epithelial cells are hypertrophic. (Hematoxylin and eosin stain, 366 magnification)

case, or occupational exposure was elicited. Furthermore, no apparent hematologic disorders were noted at the time of study with the exception of a positive sickle cell preparation.

The clinical course of this disease is well documented (1). It runs an insidious course, characterized by malaise, cough, dyspnea, and weight loss. Occasionally, the appearance of the disease is preceded by symptoms and signs of respiratory infection. There may be low-grade fever, chest pain, and, at times, productive cough. The sputum may be blood streaked. Later in the disease, cyanosis, clubbing of fingers, and pulmonary insufficiency become prominent. Bacterial and mycotic infections may occur and complicate the disease process (1, 3–5). The diagnosis of the disease is usually made by lung biopsy, as it was in this case. In one case reported by Carlson and Mason (2), the disease was tentatively diagnosed by examination of the sputum alone. In the present case the solid components of the sputum were found to contain proteinaceous material which is identical to that found in the pulmonary biopsy. It is possible that histologic sections of the sputum may disclose the presence of the characteristic alveolar exudate, and this may be useful in establishing a tentative diagnosis before the pulmonary biopsy is taken.

The pathogenesis of this disease is obscure. It has been suggested that an inherent defect of the pulmonary capillaries (6) or an immune reaction (7), in response to either exogenous or endogenous antigenic substance, may be responsible for the development of the disease. Although an infectious process may be involved, no causative agents have been demonstrated (1). Tuberculosis has been suspected in most reported cases, but there is only one documented case (8) in which active tuberculous infection preceded the development of this disease.

Despite the resemblance of the exudate of the alveolar proteinosis to pneumocystis pneumonia, no organisms have been demonstrated in the former condition. The exudate of pneumocystis pneumonia appears to be more foamy, and the cystic form of organisms are invariably demonstrated with Grindley fungous stain or Grocott-Gomori methenamine silver stain. Furthermore, the pneumocystis infection usually occurs as a terminal complication of a severe debilitating condition or of a hematologic disease (9). Plenk and associates (10) reported that four of nine patients with alveolar proteinosis demonstrated positive reactions to a rather specific pneumocystis antigen. The relationship between this disease and pneumocystic infection is not clear, however.

Since the roentgenographic changes are similar, a study was made to correlate the histologic changes of alveolar proteinosis with pulmonary edema related to a great variety of underlying diseases, such as circulatory disorders, thromboembolism, neoplasms, bacterial and mycotic infections, blood dyscrasias, and many other conditions. Five hundred and thirty-two cases of pulmonary edema found in three thousand consecutive autopsies at Duke Medical Center were reviewed. These cases were noted to differ from alveolar proteinosis in the clinical aspects, the over-all histologic features, and the basic process. It is believed that the changes seen in alveolar proteinosis are not related to pulmonary edema.

Histochemically, the material in the alveoli contains large amounts of mucopolysaccharides and lesser amounts of lipid. It resembles that seen in caseous necrosis, and may actually represent an altered inflammatory exudate or cellular debris. The possibility that it is due to abnormal protein metabolism of the alveolar or the bronchial epithelial cells awaits further investigation.

Summary

A case of pulmonary alveolar proteinosis in which the diagnosis was made by lung biopsy is discussed. In addition, a follow-up examination of the sputum was performed and correlated with the findings of the biopsied specimen. It is believed that histologic study of the sputum may be used for a tentative diagnosis of this disease. A brief review is made of the clinical pathologic features of the disease.

Sumario

Proteinosis Alveolar Pulmonar

Se discute un caso de proteinosis de los alvéolos pulmonares en el que se hizo el diagnóstico por la biopsia pulmonar. Además, se hizo un examen subsiguiente del esputo y el resultado se correlacionó con los hallazgos en el ejemplar de la biopsia. Parece que cabe usar el estudio histológico del esputo para un diagnóstico tentativo de esta do-

lencia. Se ofrece una breve reseña de las caracteclínico-patológicas de la enfermedad.

Resume

Protéinose pulmonaire alvéolaire

La discussion porte sur un cas de protéinose pulmonaire alvéolaire dans lequel le diagnostic fut effectué grace à une biopsie pulmonaire. En plus, on pratiqua une étude suivie de l'expectoration et on la rattacha aux données de l'échantillon de biopsie. On croit que l'étude histologique de l'expectoration peut être utilisée pour essayer de diagnostiquer cette maladie. Les auteurs font une courte revue des traits clinicopathologiques de la maladie.

Acknowledgment

The writers wish to acknowledge the contribution to this paper of Dr. Shao-nan Huang, Department of Pathology, Duke University.

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PRIMARY FIBROSARCOMA OF THE BRONCHUS[1, 2]

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INTRODUCTION

Primary fibrosarcoma of the bronchus constitutes one of the significant entities among sarcomas of the lung which, however, represents only a small proportion of the over-all malignancies of the lung. According to Drewes and Willmann (1), carcinoma of the lung is one to two hundred times as frequent as sarcoma of the lung. Ochsner and Ochsner (2), in agreement, record seven sarcomas of the lung as compared to 890 cases of bronchogenic carcinoma. In 1938, Mallory (3), in reviewing more than 8,000 autopsies at the Massachusetts General Hospital, found one primary sarcoma of the lung and Noehren and McKee (4) found only one sarcoma among 7,272 autopsies at the Kansas City Hospital. Donohue and his associates (5) found only one primary fibrosarcoma of the bronchus among 11,626 patients who had undergone bronchoscopy at the Mayo Clinic in the years 1945 to 1956.

Fibrosarcoma primary in the bronchus has been reported in only ten previous instances (table 1). While Drewes credits the first description of primary sarcoma of the bronchus to Poisson and Robbin in 1856, a lack of description makes it impossible to confirm many of the previously described tumors as definite fibrosarcomas. Many of the early cases reported as primary fibrosarcoma of the lung were not clearly distinguished from the undifferentiated carcinomas, particularly the so-called "oat cell" tumor. Stout (6) has detailed definite criteria for the identification of those tumors which should be considered fibrosarcomas, and this has done much to eliminate previously existing confusion. He includes only those tumors which are composed of fibroblasts, producing collagen and reticulin fibers. This latter can be demonstrated by the silver-stained network around the individual tumor cells. The individual cells are fusiform and are arranged in bundles which tend to interlace either in gentle or abrupt changes of direction to give the appearance of curves or whorls. The extent of production of collagen and reticulin depends on the degree of differentiation of the tumor. Most true fibrosarcomas are well differentiated and slow growing with well differentiated collagen and reticulin fibers, little hyperchromatism or pleomorphism, and rare mitotic figures. Less differentiated fibrosarcomas have fewer collagen and reticulin fibers and show closely packed cells with many mitotic figures with pleomorphism and hyperchromatism of the nuclei.

Grossly, the sarcomas of the bronchus have all been polypoid endobronchial tumors, with various degrees of extension through the bronchial wall into the lung parenchyma in a dumbbell fashion. Most have been reddish, lobulated, and quite vascular. The larger tumors projected along the contour of the bronchial lumen as long tongues of tissue.

Black (7) in 1949 reviewed the previously reported cases of sarcoma of the lung and was able to find only five which had sufficient histologic description to confirm the diagnosis of fibrosarcoma according to the criteria of Stout. He reported one additional case in a 46-year-old woman who is the oldest patient thus far reported. Since then, four additional cases which appear to be well authenticated have been reported. The present writers have recently identified one additional case which apparently makes the eleventh case to be reported.

CASE REPORT

P.H. (History No. 24472), a 31-year-old white man, was admitted to the University Hospital on January 22, 1960, because of three episodes of right-sided pneumonia since August, 1959. He had been admitted previously in September, 1957, for neurolysis for a sciatic nerve injury incurred in an automobile accident, with fracture of the right pubis and dislocation of the right hip. As he had no respiratory symptoms at that time, a chest roentgenogram was not taken. He had episodes of pneumonia in August, October, and December,

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[2] Supported by grant No. H-5365 from the Heart Institute of the National Institutes of Health, U. S. Public Health Service.

TABLE 1

REPORTED CASES OF FIBROSARCOMA

Author	Age, Sex, and Race	Symptoms	Therapy	Results
Baum *et al.* (10, 11)	29 F W	1½ months' cough, chest pain, weight loss	Bronchoscopy and irradiation 18,630 r. over 2½ years	Died 33 months after onset from tuberculosis.
Johns and Sharpe (9)	18 M W	2 months' cough, hemoptysis	None. Large friable tumor replacing right lung; no metastases at autopsy	Died of tumor 15 months after onset.
Lewis (14)	14 M W	1 month's cough, cyanosis, pain, dyspnea	Bronchoscopy and irradiation	Living and well with normal roentgenogram 2½ years after onset.
Mallory (3)	13 F W	2 months' cough, pain, pleurisy, weight loss	Exploratory thoracotomy	Died of intrapulmonary hemorrhage at operation.
Pollack (15)	19 M W	7 months' cough, pain, fever, wheezing, weight loss	Bronchoscopy and fulguration 5 times	Living and well 6 years later. Bronchoscopy, bronchograms and roentgenogram negative at 31 months.
Black (7)	46 F W	9 months' cough, fever, weight loss, hemoptysis, recurrent "flu"	Left pneumonectomy	Died of recurrent tumor 6 years and 5 months after operation.
Carswell and Kraeft (12)	29 M W	5 months' cough, dyspnea, pain, wheezing, pneumonitis	Left pneumonectomy	Living and well 7½ months later with normal bronchoscopy.
Curry and Fuchs (8)	13 F W	6 weeks' cough, wheezing, pain, hemoptysis	Coughed up tumor. Bronchoscopy showed no residual tumor	Living and well with normal roentgenogram 4 years and 3 months after onset.
Donohue, Andersen, and McDonald (5)	5½ F W	14 months of recurrent fever (?—rheumatic); 6 months' cough	Right pneumonectomy	Living and well 3 months after operation. Lost to follow-up since.
Holinger *et al.* (13)	5½ M W	7 months' cough, fever, hemoptysis, pneumonitis	Resection, right middle and lower lobes	Postoperative course uneventful.
Webb and Hare (present case)	31 M W	5 months' cough, fever, wheezing, pneumonitis	Right pneumonectomy	Living and well with normal roentgenogram 15 months after operation.

1959, all of which required hospitalization and gradually responded to antimicrobial drugs. In between the episodes the patient had no chest pain, fever, dyspnea, arthralgia, anorexia, or weight loss. Roentgenograms taken at each hospitalization revealed a right mid-lung infiltrate diagnosed as "virus pneumonia," "lobar pneumonia," and "pneumonitis." At the time of admission he had persistent wheezing and a cough productive of one-fourth of a cup of thick, yellow sputum daily. He had smoked one and one half packages of cigarettes daily for over ten years.

Physical examination: The patient's temperature was 99°F.; pulse, 72 per minute; blood pressure,

110 systolic, 70 diastolic in mm. of mercury; respirations, 18 per minute. He was well developed and well nourished. The right side of the chest showed a marked lag and the right diaphragm did not descend with inspiration. The right lung was dull to percussion at the base, with diminished breath sounds and respiratory wheezes. The left lung and heart were normal and the liver and spleen were not palpable. The right leg was atrophied with a peroneal palsy.

The chest roentgenograms (figure 1) showed a multilobulated, fairly well circumscribed mass in the right hilus extending inferiorly which appeared to involve both the middle and lower lobes. There were several small calcific deposits around the mass. Laminagrams (figure 2) revealed many calcific deposits in the right hilus and perihilar areas and a large, well circumscribed soft tissue mass with smooth rounded borders. This was intimately associated with the right main stem bronchus and extended out from the hilus on a broad base into the anterior portion of the lung. Distal to the mass were multiple cavities up to 2 cm. in diameter.

Laboratory data revealed a leukocyte count of 3,850 with 8 bands, 45 neutrophils, 3 eosinophils, 38 lymphocytes, and 6 monocytes. The urinalysis was negative. The hemoglobin was 15.2 gm.; hematocrit, 48.5 per cent. A fasting blood glucose was 93 mg. per 100 ml.; nonprotein nitrogen, 43 mg. per 100 ml.; total protein, 7.5 gm. per 100 ml., with 4.9 gm. albumin and 2.6 gm. globulin; prothrombin time 43 per cent of normal. Several sputum specimens and gastric washings were negative for acid-fast bacilli and fungi on microscopy and culture. The tuberculin and blastomycin skin tests were negative; the histoplasmin skin test was strongly positive. The histoplasmin complement-fixation antigen one was positive at 1 to 32; antigen two, positive at 1 to 16.

Bronchoscopies on January 28, 1960, January 30, 1960, and February 16, 1960, each revealed a large white mass covered with gliary mucus which appeared very friable and nonvascular. It almost completely occluded the right main stem bronchus with only a slitlike opening extending anteromedially and inferiorly along the main stem bronchus as if the mass was projecting up from the distal bronchus into the trachea. Numerous large biopsies were obtained at each bronchoscopy, and each revealed only necrotic tissue and exudate with a few nonspecific cells. On one occasion it was thought that the caseation with flecks of calcium was suggestive of histoplasmosis.

On February 20, 1960, a right pneumonectomy was performed. The polypoid projection of the tumor extended up past the carina but was not attached to the right main stem bronchus so that there was adequate free tissue for closure of the bronchus.

Gross pathologic findings: The lung weighed 540 gm., with aeration of the upper lobe but with evidence of pneumonitis in the middle lobe and in the anterior portion of the basilar segments of the lower lobe. The main stem bronchus

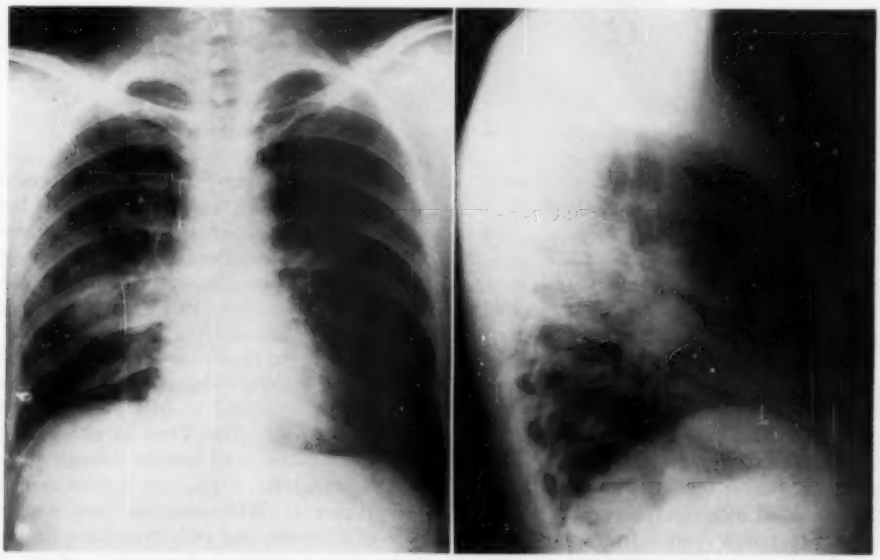

FIG. 1*A* and *B*. Posteroanterior and right lateral chest roentgenograms showing the right subhilar mass with infiltration extending into both the right middle and lower lobes.

FIG. 2. Laminagram showing well-circumscribed nature of the right subhilar mass.

was almost completely occluded by a mass of pinkish-white translucent tissue that assumed the shape of the main stem bronchus, with a branch extending approximately 2 cm. into the right upper lobe bronchus (figure 3). The mass extended free in the lumen for 6 cm. down the bronchus before infiltrating into the lung tissue of the middle and lower lobe. It measured more than 9 cm. in length, being 1.8 cm. in diameter at the top but more than 5 cm. in diameter after it extended into the middle lobe. The distal bronchioles of the right middle lobe and anterior and medial basilar segments of the right lower lobe were filled with thick, tenacious yellow-red mucus. Six lymph nodes from the specimen showed anthracosis, but no evidence of metastases.

Microscopic findings: The frozen section obtained by biopsy at thoracotomy showed large coarse bundles of fibroblasts growing in various directions. Large numbers of inflammatory cells, particularly plasma cells, were admixed with the fibroblasts. A diagnosis was made of "probably low-grade fibrosarcoma."

In the permanent sections prepared from the entire right lung the main mass of the tumor was found to be composed of cells which generally had a bipolar structure. These extended irregularly in large and small bundles through all pulmonary structures, wiping out normal architecture (figure 4). While some of these consisted only of a nucleus and pink cytoplasm, many of them showed definite collagen fibrils at the two ends of the cell. The nuclei of these cells, both

those not showing collagen fibers and to a lesser extent those with fibers, frequently showed a single pink nucleolus in a vesicular nucleoplasm. The nuclei were large and potato shaped, sometimes with an indentation in one side. Thus, most of the cells were readily identifiable fibroblasts, both young and old, undergoing fairly rapid proliferation. No definite mitotic figures were observed. The fibroblasts frequently coursed irregularly through the tissue, but in many areas they were arranged in dense bundles, having the appearance of overrunning and destroying preexistent tissues.

A Wilder's silver reticulum preparation (figure 5) confirmed the fibroblastic nature of the tumor. The fibrils also gave the typical green color of collagen with Gomori's modification of the Masson Trichrome stain. In the lung parenchyma proper, the alveolar pattern was destroyed, thus contrasting sharply with usual forms of fibrosing pneumonitis. In the bronchioles, the tumor extended to the epithelial surface but, somewhat surprisingly, usually did not cause ulceration (figure 6). The reserve cells of the mucosa commonly showed marked hyperplasia, but the columnar cells overlying them usually persisted, and retained their cilia.

In all sections of the tumor there was a surprising number of plasma cells. These were not noted to any significant degree in the lung outside of the tumor proper. While plasma cells are occasionally present in and around various neoplasms, previous reports of fibrosarcomas of the lung have not stressed their presence, a point which almost certainly would not have been omitted had they been as prominent as in the present case.

The fibroblasts exhibited a striking tendency to penetrate the bronchial walls, where the cartilaginous rings were destroyed and the normal smooth muscle bundles were widely separated and frayed. When the tumor extended into the lumen of the bronchus as a polypoid projection, ulceration was constant.

There was no sharp line of demarcation around any margin of the tumor. The fibroblasts merely blended with normal lung parenchyma, and it was impossible to state the exact point at which neoplasia ended. Only minimal pneumonitis was noted at the margins of the tumor, and many times the tumor blended with what appeared to be normal lung.

In a subpleural location, some distance from the tumor proper, were several hyalinized granulomas measuring up to 6 mm. in maximal diameter. While these were not calcified, they were

FIG. 3. Picture of the resected right lung showing tongue of tumor projecting out of the main stem bronchus and into the upper lobe bronchus.

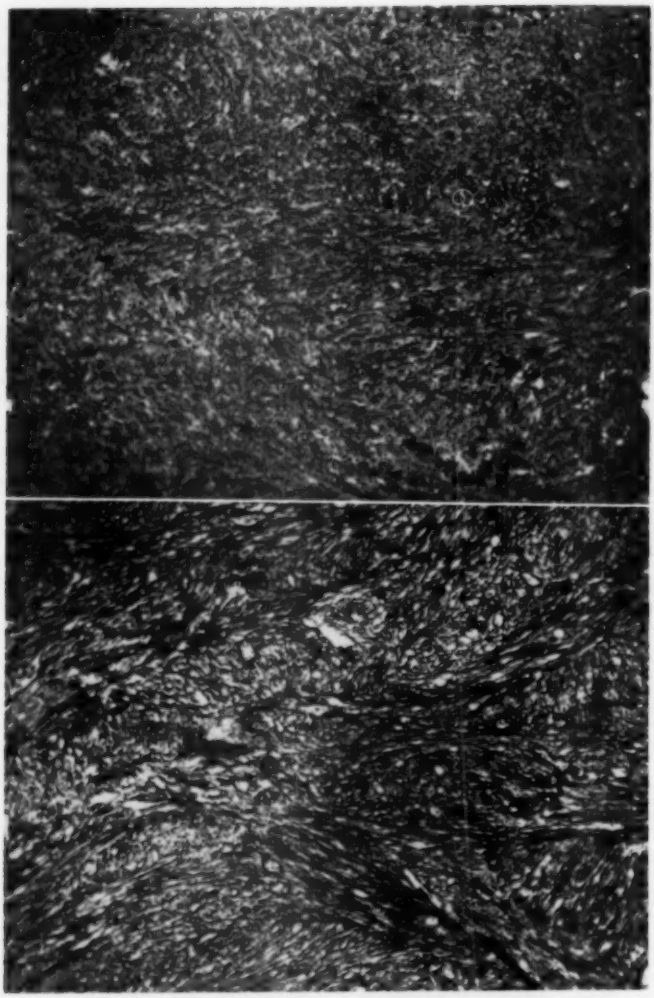

FIG. 4 (Top). Photomicrograph (×70) of fibrosarcoma showing typical coarse bundles of bipolar cells.

FIG. 5 (Bottom). Photomicrograph (×70) of Wilder's silver reticulum preparation, confirming the fibroblastic nature of the tumor.

well encapsulated with hyaline scar tissue and appeared inactive. No organisms were seen in the granulomas in the hematoxylin and eosin or acid-fast preparation, but large numbers of organisms with the classical appearance of *Histoplasma capsulatum* were seen in a Gomori's silver methenamine preparation (figure 7). After finding these organisms in the granulomas, the tumor sections, normal lung, and bronchial exudate were reviewed, but no Histoplasma at all was found outside of the granulomas.

Follow-up study: The postoperative course was completely benign and the patient was discharged on the eighth postoperative day. He has remained completely asymptomatic, is working full time, and has gained more than ten pounds since discharge. At his most recent follow-up examination on May 12, 1961, he stated that

FIG. 6 (Top). Photomicrograph (×70) showing tumor extending to the bronchial mucosa without producing ulceration.

FIG. 7 (Bottom). Photomicrograph (×450) of Gomori's silver methenamine preparation of subpleural granuloma showing typical *Histoplasma capsulatum* organisms.

he had minimal dyspnea on severe exertion but much less than prior to operation. His physical examination and roentgenograms showed no evidence of recurrence of the fibrosarcoma.

DISCUSSION

Noehren and McKee, in a survey of thirty-five cases of all types of sarcoma of the lung, found the highest age incidence in patients in the third decade and the second highest in patients between fifty and sixty years of age. According to Stout, fibrosarcoma of all sites or origin appears to be a disease of young people, with the greatest frequency in the third decade of life. Fibrosarcoma of the bronchus appears to involve primarily the younger age group: two

of the patients were five and one-half years of age, five were in their teens, two were twenty-nine, one was thirty-one, and the oldest was forty-six years of age. There appears to be no predominant sex distribution, as five were females and six were males.

Fibrosarcomas of the bronchus are of clinical importance, not only because of their malignant nature, but because of their location and obstructive potential. The symptoms are principally those of obstruction. Cough, usually productive, was present in every one of the reported cases, and most patients have had repeated episodes of lower respiratory infections. Wheezing was frequent, and hemoptysis is reported as having occurred in at least four of the patients.

The roentgenographic appearance in virtually all cases has been that of a pneumonia and this has usually been the initial diagnosis, although several observers have recognized that the lobar or segmental nature of the lesions suggested a pneumonitis distal to an obstruction.

It is significant that, in nine of the reported cases, bronchoscopies were performed, and in eight instances the tumor was demonstrated. In the case of Curry and Fuchs (8), bronchoscopy revealed the apparent site from which the tumor had been expectorated although no residual tumor was demonstrable. In the autopsy case reported by Johns and Sharpe (9), bronchoscopy had not been performed, but from the description of the tumor it could have been visualized by bronchoscopy.

Most of the tumors have arisen from the main stem bronchi or in a lobar bronchus and have projected outward in a polypoid fashion, showing a tendency to grow into and along the bronchial lumen. The present case grew thus as the tongue of projecting tissue extended 6 cm. up the bronchus from its site of origin and bifureated into the upper lobe bronchus. It is significant that in describing 14 cases of endobronchial tumors other than bronchogenic carcinoma, Donohue, Andersen, and McDonald (5) found one case of fibrosarcoma of the bronchus and this was the only malignant tumor of the bronchus of non-carcinomatous origin. All the others were benign though, nevertheless, important clinically because of their obstructive nature.

The treatment of these patients has been most unusual as all forms of therapy have been tried with surprising success. As these tumors are slow growing, it is possible that the ten-year follow-up study will be more significant as to ultimate fate than the five-year follow-up. Expectoration of the tumor was apparently curative in the case of Curry and Fuchs (8), as this patient was still living and well with a normal roentgenogram four years and three months after onset of her symptoms. Two of the patients were treated by bronchoscopic removal of the tumor followed by irradiation. One of these died of tuberculosis thirty-three months after onset with no evidence of residual tumor at autopsy. The other was living and well with a normal roentgenogram two and one-half years after the onset. Another, treated by bronchoscopy and fulguration, which in general would seem to be poor therapy, was still living and well six years later, having had completely negative bronchoscopy, bronchograms, and roentgenographic findings thirty-one months post onset. Six of the patients had been subjected to operation, one dying at operation from intrapulmonary hemorrhage from the tumor, and another dying of metastases six years and five months after pneumonectomy. Three others had pneumonectomies, and one a right bilobectomy, with good results after a limited follow-up period. In view of the dumbbell nature of this tumor which projects both intraluminally and intraparenchymally, bronchoscopic removal would not seem to be the treatment of choice. Total excision of the lesion appears to be the best therapy and probably should be limited to lobectomy whenever this is sufficient to totally circumscribe the local lesion.

Summary

The eleventh confirmed case of fibrosarcoma of the bronchus is reported. This lesion presents a definite clinical entity, with symptoms of bronchial obstruction and a tumor which can always be demonstrated bronchoscopically. Even though cures have been reported by irradiation, fulguration, and expectoration of the tumor, it is believed that limited pulmonary resection is the treatment of choice for these lesions.

Sumario

Fibrosarcoma Primario del Bronquio

Se presenta el undécimo caso confirmado de fibrosarcoma de un bronquio. La lesión constituye una entidad clínica bien definida con síntomas de oclusión bronquial y un tumor que puede observarse siempre broncoscópicamente. Aunque se han

descrito curaciones con la irradiación, la fulguración y la expectoración del tumor, parece que la resección pulmonar limitada es el tratamiento de elección para estas lesiones.

Resume

Fibrosarcôme primaire des bronches

Les auteurs rapportent le onzième cas confirmé de fibrosarcôme des bronches. Cette lésion présente une entité clinique nette avec des symptômes d'obstruction bronchique, et une tumeur qui peut toujours être prouvée à la bronchoscopie. Bien qu'on ait rapporté des guérisons par irradiation, electrocoagulation, et expectoration de la tumeur, on croit qu'une résection pulmonaire limitée est le traitement de choix.

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PERIPHERAL NEUROPATHY DUE TO ETHIONIAMIDE[1]

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INTRODUCTION

Various difficulties may be attendant upon the use of new drugs. Some quickly reveal themselves, while others, perhaps due to affect only a small minority of patients, manifest themselves much later (1). While using ethioniamide recently such a problem was encountered, and this case report is published in the hope that it may contribute to a fuller understanding of the properties of this drug.

CASE REPORT

J.W., aged fifty-five years, was admitted to the hospital on March 3, 1959, with an exacerbation of long-standing pulmonary tuberculosis first diagnosed in 1947. Tubercle bacilli were present in his sputum on both microscopy and culture.

The patient had several periods of treatment in sanatoriums and hospitals and antituberculosis drugs were given from 1950 in a variety of courses. In 1952 isoniazid was used alone, and as a result the patient's organisms became resistant to this drug. The organisms subsequently also became resistant to streptomycin, probably due to the fact that the patient did not take para-aminosalicylic acid (PAS) regularly. During the year 1957 isoniazid was given alone in the relatively large dosage of 600 mg. daily. This was done in the face of known resistance to isoniazid in the hope that, as has been suggested, large doses of isoniazid given alone in such cases might still be effective. Isoniazid therapy with such a massive dose has since been shown to be of questionable value (2).

In February, 1959, the patient's general condition deteriorated. He complained of considerable lassitude, increase in the cough and sputum previously present, and a chest roentgenogram showed considerable extension of the disease on the left side. His sputum contained tubercle bacilli, still resistant to streptomycin and isoniazid. This, together with the patient's refusal to take PAS, led to treatment with two of the newer drugs, to both of which the tubercle bacilli were known to be susceptible. This treatment, comprising 1 gm. of ethioniamide and 2.5 gm. of pyrazinamide daily in divided doses, was commenced in March, 1960.

There was an excellent response to the treatment. The patient's pyrexia subsided, his weight increased, and sputum diminished. There was satisfactory roentgenographic improvement, and sputum cultures became negative for a time. Serial liver function studies showed no toxic effect resulting from the use of pyrazinamide. The usual hyperuricemia occurring with the administration of this drug was noted (3). After two months of treatment the patient complained of shooting pains in both feet, a sensation of swelling and numbness about the soles of the feet, and occasional tingling in the toes and fingers.

On clinical examination no abnormal signs were found, and roentgenograms of the hands and feet showed no abnormality. The symptoms gradually increased. Early in August, 1959, antituberculosis chemotherapy was temporarily suspended, and it appeared that the symptoms regressed. With the resumption of treatment, however, the symptoms again increased and the patient described a burning sensation in both hands and feet. At this time the patient had become depressed and somewhat garrulous and argumentative. Examination of the calves, feet, and toes showed a tenderness to pressure, and he experienced distressing dysesthesia which persisted for many seconds after the stimulus had been removed. There was considerable impairment of vibration sense from the iliac crest downward. Joint position sense in the feet was impaired. The knee jerks were exaggerated and the ankle jerks were absent. The severe dysesthesia made it impossible to assess his plantar responses. There was considerable tenderness of the fingers with some hypalgesia. The arm reflexes were all brisk.

Lumbar puncture showed clear colorless cerebrospinal fluid under normal pressure, the protein content being elevated to 80 mg. per 100 ml., with no increase in cells. His electroencephalogram was normal. The serum B_{12} level was within normal limits. Treatment with all drugs was suspended and the patient was given oral pyridoxine and pantothenic acid.

Since the suspension of pyrazinamide and ethioniamide in September, 1959, there has been slow, but progressive improvement. The patient's mental state has improved and the signs of peripheral neuritis have substantially regressed. There are no abnormal signs now in the upper limbs. Vibration sense is still depressed over the toes, but is normally appreciated at the ankles. Postural sense is depressed only in the toes. Sensation over the legs is normal, although some hyperalgesia and hyperesthesia over the dorsum of the feet remain. The ankle jerks are still absent. The plantar response is now clearly ex-

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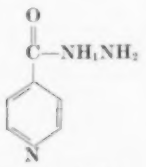
tensor, suggesting a myelopathic component to the recent neuropathy. The protein content in the cerebrospinal fluid has reverted to normal.

Comment

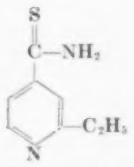
The patient is considered to have a toxic peripheral neuropathy, presumably due to ethionamide. When his treatment was first suspended, his symptoms rapidly regressed, but recurred more severely when treatment was again instituted three weeks later. Ethionamide therapy was therefore stopped, but this time symptoms and signs of neuritis were much slower in resolving. Twelve months later, despite continuing improvement, some residual sensory deficit remains.

Neurotoxic effects, possibly due to ethionamide, have been previously reported by Brouet and associates (4). They recorded four cases, but in none of these was ethionamide shown to be the definite cause. Convulsions were observed in two of their patients under treatment with ethionamide and cycloserine, and the latter drug could presumably have been responsible for their production. In the third patient, who had previously received isoniazid in high dosage for a great length of time, symptoms of polyneuritis developed during combined therapy with isoniazid and ethionamide. A fourth case is recorded in which treatment with isoniazid was complicated by symptoms of neuritis which improved when the drug was suspended. Within three days of commencing treatment with ethionamide these symptoms reappeared, only to disappear once again after this drug was suspended.

Both isoniazid and ethionamide are derivatives of isonicotinic acid and there are some chemical similarities between the two compounds.



Isoniazid



Ethionamide

Studies of ethionamide analogues have shown that the ethyl group in ethionamide enhances activity, but the simpler compound with no ethyl group has a qualitatively similar type of antituberculous activity. Isoniazid is a hydrazide of isonicotinic acid, and ethionamide is a thioamide of this acid. The absence of cross resistance between isoniazid and ethionamide, despite the chemical relationship between these substances, would suggest that the underlying mechanism of antimicrobial activity is different. In fact, ethionamide has an action which may resemble that of the thiosemicarbazone derivative, thiacetazone. From this it would seem that the thioamide group is more significant in the antituberculous action of ethionamide than the fact that it is a derivative of isonicotinic acid. Although isoniazid and ethionamide are derived from the same product and there are some pharmacologic analogies between them, toxic reactions to these drugs are not necessarily similar when they are successively administered to the same patient. In the present case large doses of isoniazid alone had previously been administered for a whole year without the development of any toxic symptoms.

Pyridoxine has been shown to prevent the emergence of peripheral neuritis due to isoniazid toxicity (5), and it has been suggested that it may be similarly effective when used with ethionamide. Certainly, the drug should be suspended at the first sign of any neurotoxic effect.

Cases of isoniazid toxicity have been reported in which a confusional psychosis and a peripheral neuritis coexist (6). It has been suggested that cases of psychosis and neuritis which occur together in the same patient represent two different types of toxicity due to isoniazid. This view is supported by the number of cases reported (7) in which pyridoxine appears to have prevented the emergence of peripheral neuritis, while not protecting the patients against the development of a toxic confusional psychosis.

Without deserving the description of a toxic confusional psychosis, the patient of the present case evinced a definite mental change with depression, mild confusion, and excitement, gradually reverting to his former mental state several months after ethionamide had been suspended. Such a mental change following the administration of ethionamide has not previously been reported, and it seems likely (as is the case with isoniazid) that it and peripheral neuritis constitute different types of toxicity which may, however, occur together.

Summary

A case of peripheral neuropathy due to ethionamide is reported. A mild toxic confusional psychosis appeared to develop simultaneously. Toxic reactions tend to be specific for particular substances, and ethionamide may thus evoke a toxic reaction in patients to whom isoniazid has previously been given without untoward effect. Ethionamide should be suspended at the first sign of any neurotoxic effect. Peripheral neuropathy may continue to improve over a long period, and some residual neurologic deficit may remain.

Sumario

Neuropatía Periférica Debida a la Etioniamida

Preséntase un caso de neuropatía periférica debida a la etioniamida. Pareció presentarse simultáneamente una leve psicosis tóxica de confusión. Las reacciones de toxicidad tienden a ser específicas para sustancias dadas y la etioniamida puede por esto provocar una reacción tóxica en enfermos a quienes se ha administrado antes isoniacida sin efecto desfavorable. Al primer signo del menor efecto neurotóxico, debe suspenderse la etioniamida. La neuropatía periférica puede continuar mejorando durante un período prolongado y también puede quedar un déficit neurológico.

Resume

Névrite périphérique due à l'ethioniamide

Les auteurs rapportent un cas de névrite périphérique due à l'ethioniamide. Il sembla qu'une forme peu grave de psychose confusionnelle toxique se développa simultanément. Des réactions de toxicité tendent à être spécifiques de certaines substances et l'ethioniamide peut ainsi provoquer une réaction toxique chez des malades à qui on avait donné auparavant de l'isoniazide sans effet contraire. L'ethioniamide devrait être arrêté au premier signe d'effet neurotoxique. La névrite périphérique peut continuer à s'améliorer pendant longtemps, et quelques séquelles neurologiques de déficit peuvent persister.

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Editorial

The Big Push Ahead in Tuberculosis Control[1]

The disease, by whatever name it has been called —consumption, "the white plague," tuberculosis— is retreating in America. The decade ahead of us, the sixties, will be decisive. We are determined, all of us here and many others, that the retreat of the tubercle bacillus shall inflict as few casualties upon our human resources as possible.

To ensure that goal, the Arden House Conference on Tuberculosis was held late in 1959. A major recommendation of that Conference was to make more widespread use of the antituberculosis drugs. The conferees at Arden House knew that to call for wider use of these drugs is one thing, but to develop realistic plans for accomplishing the task is something else again.

The unvarnished truth is that tuberculosis workers have found it increasingly more difficult to find the persons who have active tuberculosis today. It was relatively easy to apply mass measures when the problem was massive; it is impractical to apply those same measures when the tuberculous are few and scattered. In other decades, we could use "shotgun" case-finding methods and mow down the tubercle bacilli in huge numbers. Today, the character of tuberculosis control work requires case-finding methods to have the accuracy of high-powered rifles. Where we could once meet the tubercle bacillus in open, pitched battle, we must now seek out an enemy that has retreated into more elusive terrain.

Let me illustrate what I mean: Many persons who have tuberculosis today are of a type which I characterize as "quicksilver cases." These persons are known by local health officers to have active, infectious tuberculosis, but they are a here-today-gone-tomorrow kind of person. Many resist any treatment at all; others begin treatment, then discontinue it too soon. Some will not accept hospital care; others go to a hospital but leave before treatment is complete. Worse still, many of these people slip out of the extensive tuberculosis surveillance network. What happens to these people is a large and costly question. It costs our nation about $700 million to live with the tubercle bacillus each year. We can ill afford such expense when we know the disease is preventable.

In upstate New York in 1959, about 13 per cent of the nonhospitalized tuberculous who needed medical treatment were without medical supervision. Tuberculosis simply cannot be defeated in this kind of situation. In spite of all our efforts some persons with tuberculosis will live at home; they must be kept under medical suprevision and, when indicated, receive adequate drug treatment. The number of these people is small, but their unwitting capacity to spread disease and misery is large indeed.

In the face of these new problems in tuberculosis control, we have changed our public health strategy in upstate New York. The change is primarily one of emphasis and intensification of efforts in all phases of prevention, diagnosis, treatment, and rehabilitation. For example, case finding has two primary objectives that inevitably lead to prevention of occurrence of new cases and to minimizing the ill effects of disease already present. The first is to find new cases of active tuberculosis in our population and to get these people under regular, supervised treatment. This means hospitalization at the outset and until the disease is no longer considered infectious.

The second objective is to seek out all persons in close contact with these newly discovered cases, to examine them promptly, and to get the active cases under medical care and the others under follow-up supervision.

As you know, we no longer have a separate Division of Tuberculosis Control in our State Health Department; instead, tuberculosis control work is now done in our new Division of Chronic Disease Services. You are also no doubt aware that whereas we once had seven state tuberculosis hospitals we now have three. In the past five years, four tuberculosis hospitals have been converted to other uses. All of these measures do not mean that we have lost interest in tuberculosis control work. Quite the contrary, we are as concerned with it as before, and possibly more excited about it because we have the opportunity of knocking out tuberculosis if we mobilize our forces properly. However, the nature of the tuberculosis control problem today is such that we no longer find it economical to have large numbers of people working to find a small number of cases through mass surveys, nor do we need nearly the number of tuberculosis hospitals as before. A glance at the tuberculosis incidence charts of the past few decades tells this story eloquently.

[1] Presented by Dr. Brightman at the Annual Conference of the New York Tuberculosis and Health Association, Inc., and the State Committee on Tuberculosis and Public Health of the State Charities Aid Association, New York, New York, April 27, 1961.

In the past, many persons with active tuberculosis were found by two methods. The first was general community roentgenographic surveys, and the second was obtaining routine roentgenograms of persons more than fifteen years of age admitted to general hospitals. In both methods, initial findings were followed up by the local health departments or in chest clinics conducted by tuberculosis hospitals.

These mass survey methods appeared effective when tuberculosis was more prevalent, but in 1959 and 1960 we thought it was time their effectiveness was critically reviewed. So we gave one of our epidemiologists the job of evaluating our tuberculosis case-finding activities, and this is what he found: The number of cases found by mass survey methods among a similar number of persons examined had dropped from 454 in 1952 to 187 in 1958. During this seven-year period, the cost of finding a person with active tuberculosis by our two methods had jumped from $492 to $1,441 per tuberculous person found. (There were obvious differences, of course, in the relative yields and costs of the two methods.)

Our evaluator then took a close look to see whether surveys of some groups in the population turned up fewer active cases than others (we are talking specifically about active tuberculosis diagnosed by clinical and laboratory means and not just roentgenograms). The answer was, yes, some did. Surveys of students in high schools and colleges turned up the fewest—only 12 persons among 79,076 surveyed at a high cost of $3,275 per active case found.

Declining yields were also found among the general population, agricultural fair-goers, and employee groups. These findings indicated to us that we could get better results if we carefully selected the groups to be examined roentgenographically. Our evaluator then found one promising clue in his hunt for a population group with a higher yield of undiscovered tuberculosis.

His clue came when he compared two separate roentgenographic surveys of State employees—one of New York City residents, the other of upstate residents. The active cases per 1,000 found in New York City were 1.1; upstate, they were 0.2. This suggested that more urban surroundings contained more tuberculosis. Similar comparisons between more urban and less urban areas showed the same picture. Admission films in large hospitals in areas of 80,000 or more population showed consistently higher yields than those in small hospitals in less populous areas.

Then another clue was turned up, not unexpectedly. This one concerned age groups. In 1958, new cases of tuberculosis found among males fifteen to twenty-four years old in upstate hospital surveys numbered only 0.2 per 1,000. However, for males aged twenty-five to thirty-four, the figure was one per 1,000. Females in these two age groups had lower rates also. But in New York City the figures were significantly high even for the fifteen- to twenty-four-year group, the rate being 2.2 per 1,000, or higher than the figures for older group upstate.

When the figures were all in, one thing was clear: We were not getting full value for resources expended; as a matter of fact, less than 10 per cent of new active cases reported in 1959 came from general hospital and community surveys. The rest came from chest clinics and private practitioners. As noted before, between 1952 and 1957, the average variable cost of finding a previous unknown active case of tuberculosis increased from $492 to $1,441. To correct matters, this is what we did in upstate New York:

We discontinued all roentgenographic screenings of low-yield populations. This included students, State employees, persons living in areas with less than 20,000 population, and persons less than twenty-five years of age in upstate New York.

We intensified our roentgenographic screening among high-yield populations. This meant areas of more than 80,000 population.

Finally, we intensified our use of central registers for cases of active and inactive tuberculosis to give us better control of cases and contacts, and to give us a current picture of progress.

The film surveys going on today are designed on the basis of our recent evaluation, and the policies followed have been discussed jointly and agreed upon by the medical leaders of our official and voluntary agencies. We hope to concentrate greater efforts and resources on contact examination of active cases and to keep score at intervals to see our hits, runs, and errors. This kind of score-keeping is too often left out of case-finding work. We need to find out what value we are getting for monies spent. As most of you know, contact examinations can produce as high as fifty times more new cases as community surveys.

All local and district health officers in upstate New York have records of tuberculous persons in their jurisdictions. These records also list the persons in close contact with the tuberculous. What must be done is to keep control activities up-to-date by visiting the homes of the tuberculous, seeing that they keep to their drug treatments, periodically examining those with whom they are in contact, and making sure that new cases found by contact examination get prompt and full treatment.

The tuberculosis experts in our state and local hospitals believe today, as in the past, that every person with active, infectious tuberculosis should be isolated and treated in a facility set up for that purpose. I believe we should follow this advice. The

activity status of these hospitalized persons requires current evaluation; discharge is in order as promptly as possible for the benefit of both the patient and the community. Our three state tuberculosis hospitals have recently installed a check-up system so that uniform follow-up is accomplished with a minimum of effort. Their report for the quarter ending December 31, 1960, shows that the following percentages of patients had active tuberculosis: Homer Folks, 99 per cent; Mount Morris, 94.5 per cent; Ray Brook, 92.1 per cent. This is certainly optimal use of hospital facilities for active and infectious cases of tuberculosis.

A one-year survey was made a few years ago of the social and economic problems of patients at the State Tuberculosis Hospital at Ray Brook. It showed that 116 of the 263 patients then in the hospital would probably become welfare clients when they were discharged. Eighty-one were considered nonemployable by a counselor. About 52 of the patients would have to change their occupations, and needed rather intensive re-training.

Among the 182 employable patients, 10 were chronic alcoholics; 2 others had histories of drug addiction. Ninety of the employable patients had less than a high school education, and 85 were classed as semi-skilled or unskilled; such workers are usually the last to be hired and the first to be laid off in industry. Fifty of these 85 employable patients were more than forty years of age, and you know what often happens to this age group in the scramble for jobs. About 41 per cent of the total group were fifty years of age and older. Many of these older patients had other illnesses—heart disease, hypertension, diabetes, arthritis, defective hearing, and other degenerative conditions.

We need to make every effort to bring social and vocational counseling to these persons with multiple handicaps. To do this, we need to work closely with other private and official agencies. I mention the social problems involved in caring for these patients because I think too often we forget that when we fight tuberculosis we fight far more than a germ; we do battle with a way of life and, unless we can do something to change the way of life some patients will return to, the over-all gain will be small indeed. This is why it is so very important that the local units of the State Committee on Tuberculosis and Public Health lend the gentle human touches that can help the arrested tuberculous patient return to a decent, tolerable existence. To find and to treat are good, but not good enough; we need also to restore and make whole again—physically, emotionally, and socially.

At the outset of my talk, I mentioned that the Arden House Conference on Tuberculosis had set broad priorities for tuberculosis control. At the same time, the conferees urged every state health department to set its own intermediate goals and standards. These state blueprints are to be made available to the Public Health Service and the National Tuberculosis Association to help these two national groups set realistic national goals and standards.

As a starting point, a committee of the Arden House group sent out suggested intermediate goals to state health departments. As I mention the Arden House suggestions to you, I will also give you the intermediate goals that we in the State Health Department have adopted for New York.

The first intermediate goal suggested by the Arden House group was that the rate of new active cases of tuberculosis be reduced to 10 per 100,000 by 1970. The present national rate is 33 per 100,000. Our 1960 rate of new active cases in upstate New York was 27 per 100,000, a provisional figure, but close enough; in New York City, the provisional figure was almost 64.

The question now arises: Can we meet the suggested 1970 goal of 10 per 100,000? Upstate, I think we can. The average decline upstate in the past five years has been more than 10 per cent. If we can continue at this rate, we will meet the 1970 intermediate goal of 10 per 100,000. New York City has a far tougher job and a far tougher problem. The decline rate there has been considerably less than 10 per cent in the past five years; in 1957 it actually increased 5.1 per cent.

The second intermediate goal the Arden House Committee suggests is this: "For the community, control of the spead of infection to the point where not more than one per cent of the 14-year-olds react to tuberculin." We think that this Arden House goal is feasible by 1970 in upstate New York.

The next recommended goal of the Arden House Committee is to get a complete diagnosis of at least 75 per cent of the people with suspicious chest films. As you know, a suspicious roentgenogram does not mean a diagnosis of tuberculosis. All that it tells us is that a careful "work-up" is needed. We think that the Arden House goal of achieving definite diagnoses in 75 per cent of suspected tuberculous persons is too low for New York. When we do mass roentgenographic screening of communities and special groups we are able to get satisfactory follow-up diagnoses of 85 per cent of the suspected cases. In our hospital admission film program, the figure is 75 per cent, but we are not satisfied with this performance.

The next standard of the Arden House Committee is this: Within two months after a tuberculin survey, at least 90 per cent of the tuberculin reactors should receive chest roentgenograms. We think this is a reasonable goal for New York.

The Arden House group next recommends that all persons in close contact with those newly dis-

covered to have active tuberculosis be examined for the disease. They say that these examinations should be completed by January 31 of the year following their reported discovery. We think this is too long a delay for New York. A person known to be in close contact with someone who has active disease ought to be examined within three months of the discovery. We accept that 90 per cent is a reasonable proportion to examine, but we think that the examination ought to be done within three months. In the mobile population we are dealing with today, too many people move away or become lost to surveillance before we get to them.

The Arden House Committee's next suggested standard is that at least 75 per cent of the people with newly reported active tuberculosis and with sputum positive for tubercle bacilli be treated so as to reverse their status to noninfectious within six months. We think that 75 per cent is too low. Experience indicates that reversal of infectiousness in 90 per cent of these people within six months is possible when today's drugs are skillfully administered.

The Arden House Committe also recommends that, at any given time, 90 per cent of the persons known to have active tuberculosis be in a hospital or under drug treatment elsewhere. They also believe that at least 80 per cent of the people living at home with active tuberculosis should have had sputum examinations within the preceding six months. We will need to improve our record to accomplish this.

The intermediate goals we have adopted for this decade are all feasible. It is our firm intention to pursue them jointly, with the voluntary tuberculosis associations, at every level of government and group living.

Let me emphasize one point: Tuberculosis control is primarily a local task. It must be done locally because that is where the problem exists. The State Health Department has the specialists who can give sound guidance in tuberculosis control, but they can work only through local groups. The public health nurses, the general practitioners, and the chest specialists make up the key group in the vast job of follow-up services.

I think that the way to put spark into tuberculosis control work today is to organize Arden House–type conferences on a county and large city basis. The health officer should take the lead and work hand-in-hand with the executive secretary of the tuberculosis and health associations and the nearest tuberculosis hospital director; determine the extent and characteristics of the local problem; inventory the resources; evaluate each of the control activities and record the results in quantitative terms. In this way the deficiencies will show up. Then it is possible to do something about them, systematically and on a time schedule. Actually, there is nothing new about tuberculosis control in the big push ahead. The same principles and procedures hold true, but we need to change our priorities and concentrate our efforts where they will do the most good. We need to apply modern business methods to tuberculosis control and make certain that we are receiving epidemiologic values for both public and private funds being spent. This requires the most skillful program planning, execution, and continuing evaluation.

A word or two about tuberculin testing should be added at this point. The State Health Department and the State Committee on Tuberculosis and Public Health have made a joint statement to guide tuberculin-testing practices in the State.

These are the main points emphasized in that statement: In the clinical testing of individuals, the tuberculin test is a valuable tool in differential diagnosis. When a person with active tuberculosis is discovered, all household contacts and other close associates should receive a tuberculin test, and reactors should be examined roentgenographically. Nonreactors should be retested within six months and at intervals thereafter, as the physician determines. Those who change from nonreactors to reactors, especially children less than five years of age, should be considered as candidates for chemotherapy. The tuberculin test is a necessary step prior to BCG vaccination.

Analysis of mass tuberculin testing as a case-finding procedure indicates that active tuberculosis has seldom been found by routine tuberculin testing among school children or among the household contacts of children who are positive reactors. Some exceptions to this may be found in schools located in low-income areas or wherever the prevalence of tuberculosis is high.

When active tuberculosis is found in a student or in a school employee, it is recommended that any student in direct contact with the active case be tuberculin tested and chest films obtained of positive reactors. Testing should be deferred if the student has been exposed to the active case recently. Cost records should be kept in any tuberculin testing of school groups. By doing this, it is possible to determine the cost of finding a new active case. The TUBERCULIN TESTING HANDBOOK published in 1958 by the National Tuberculosis Association is a sound guide for designing records and reports.

The tuberculin test is sometimes worth using to sample the prevalence of tuberculous infection in a group or a community. Testing and retesting of random samples of the same population at appropriate intervals shows the trends in levels of infection. Used in this way, the tuberculin test can

be a useful tool in evaluating control work. A reasonable approach is to test samples of the school population on entrance to school, and again during adolescence or upon leaving school. Sampling for the prevalence of tuberculosis should be scheduled periodically within each region of the State.

When mass tuberculin testing is done, for whatever purpose, individuals who show reactions of 10 ml. or more should be followed up. Studies show a strong correlation between the size of the tuberculin reaction and the risk of developing active disease later on. The Mantoux test is preferred. PPD is recommended for Mantoux or intradermal tests; the dosage is 5 TU when a single test is used.

In interpreting tests, it is best if at least two persons make independent interpretations. The largest transverse diameter of induration should be measured in millimeters and classified in one of four groups—zero to 4 ml., 5 to 9 ml., 10 to 19 ml. and, finally, 20 ml. or more. The Mantoux test should be read at forty-eight hours to seventy-two hours. A positive test is usually 5 ml. or more of reaction. For the Heaf test, four or more indurated points are considered a positive reaction. The test should be interpreted five to seven days afterwards.

* * *

Now I would like to touch briefly on some of our current efforts to understand other lung diseases. Chronic bronchitis and emphysema seem to be on the upswing in our nation. In England these diseases comprise the third leading cause of death. No one really has learned the reasons why. The finger of suspicion has been pointed at several possible factors. Air pollution is one of the suspects. Indirect associations have been found between air pollution and various lung diseases, but of direct knowledge we have far too little.

Public support for air pollution control work has been less than it would have been if we had direct proof that polluted air causes specific ill effects to the lungs. Plans to attack this problem are now in process under the auspices of the State and Erie County health departments. A study of the department-supported Chronic Disease Research Institute in Buffalo will try to determine whether any link exists between certain kinds of air pollution and breathing capacity. Nineteen air-sampling stations to be positioned around the Buffalo area will collect air to find out which areas have high and low levels of two common pollutants—sulfur dioxide and solid particles. While this is being done, vital lung capacities are being measured among a random sample of people. These results will be correlated with the findings of high and low pollution areas. This study marks a pioneering effort in understanding the health effects of air pollution in our State.

The Chronic Disease Research Institute also set up an Emphysema and Chronic Bronchitis Research Clinic in September of 1959. A major focus of work in this Clinic has been to develop better methods of measuring pulmonary efficiency. Related studies are analyzing the effects of cigarette smoking, air pollution, occupational exposure, and family history of these two conditions.

The main point I wish to make is that the State Health Department is greatly concerned about the effect of chronic pulmonary diseases on the health of our people. At the same time that we are giving special attention to the retreating tuberculosis menace, we must also give special attention to the smoldering problems of other chronic lung diseases. This is especially important because of the increased prevalence of these diseases among older persons and the increasing proportion of older persons in our population.

* * *

This is a brief review of what we are thinking about and what we are doing in tuberculosis control and in the study of other lung diseases in the state and local health departments.

It is very good that those of us in public health and clinical medicine can get together with you who give freely of your time and effort in the tuberculosis and health associations. It is good for members of our two organizations to have an opportunity at our annual meetings to exchange views on problems of mutual concern. This exchange of ideas and plans has been particularly fruitful this year through special committees set up jointly to study program evaluation, hospital care, and tuberculin testing. What we want to do now is to put these joint plans into action in every community in the State, and get together at this time next year to report our results.

The age of chronic diseases is now upon us. The great killers and disablers of today are unquestionably heart disease, cancer, and strokes. We know so little about the big three, and all the while they take such a massive toll in human welfare. This battle to learn about the so-called degenerative chronic diseases can make many people forget that there is an important chronic disease that we can bring under control if we want to expend the time and effort and money, and I'm referring to tuberculosis. You know tuberculosis is often looked upon as mainly a communicable disease, but it also is very much a chronic disease, especially among older persons. Like many of our radioactive materials, it never quite dies out in a lifetime. So the person with tuberculosis accepts this fact of life, learns to live with his disease, observes health rules meticulously, and remains under the continuing care of his physician. By following the rules, many of our tuberculous persons live to ripe old

ages and live well in the process. This is the individual goal in tuberculosis control that one never loses sight of.

I think if we work hard in the decade ahead tuberculosis is one disease that we can relegate to a position of minor importance in public health. It will cost millions of dollars to fight tuberculosis in our State this year, and it will cost millions of dollars a year for some time to come. But recent history is pointing the way to its inevitable demise. We have only to intensify the attack with intelligence, determination, and our traditional concern for the well-being of the individual. It is this humane concern that has brought us together here today.

HERMAN E. HILLEBOE
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July 27, 1961

Recommendations for Vaccination Against Influenza in the Civilian Population

ADVISORY COMMITTEE ON INFLUENZA TO THE SURGEON GENERAL
OF THE U. S. PUBLIC HEALTH SERVICE[1]

(July 31, 1961)

Experience with Asian influenza between 1957 and 1960 has served to re-emphasize that patients in certain disease categories who acquire influenza are at much greater risk of death or severe morbidity than the normal population or patients with miscellaneous diseases. In order to reduce the hazard to patients at high risk, it is recommended that they be immunized with polyvalent influenza vaccine as soon as practicable after September 1 and no later than the beginning of the usual influenza season in late December. Since a two-week delay in the development of antibodies may be expected, it is important that immunization be carried out before epidemics occur in the area.

The adult dosage recommended by the Advisory Committee for primary immunization is 1.0 ml. (500 CCA units) of polyvalent vaccine administered subcutaneously. It is urged that persons who have not previously been immunized should also receive, if feasible, a second dose of 1.0 ml. approximately two months after the first injection. This second dose will serve to protect the small but significant proportion who do not develop adequate antibody after the first injection. Persons previously immunized should be reinoculated with a single "booster dose" of 1.0 ml. subcutaneously each year.

Patients in the following disease categories have experienced the highest mortality rates and, therefore, specific protection is clearly indicated for them as a routine practice.

(*A*) Persons of all ages who suffer from chronic debilitating disease, e.g., chronic cardiovascular, pulmonary, renal, or metabolic disorders; in particular:

(*1*) Patients with rheumatic heart disease, especially those with mitral stenosis.

(*2*) Patients with other cardiovascular disorders such as arteriosclerotic heart disease and hypertension, especially those with evidence of frank or incipient cardiac insufficiency.

(*3*) Patients with chronic bronchopulmonary disease, for example, chronic asthma, chronic bronchitis, bronchiectasis, pulmonary fibrosis, pulmonary emphysema, pulmonary tuberculosis.

(*4*) Patients with diabetes mellitus.

(*5*) Patients with Addison's disease.

(*B*) Pregnant women.

(*C*) All persons more than sixty-five years of age.

In years of expected high incidence, vaccination *is recommended* for the following groups of people:

(*1*) Persons employed in medical and health services, public safety, public utilities, transportation, education, and communications.

(*2*) Persons in age groups in which influenza occurs in highest incidence, namely, five to twenty-five years.

Dose and Schedule of Vaccination by Age

(*1*) *Adults (persons thirteen years of age or older):* 1.0 ml. (500 CCA units) should be administered subcutaneously as soon as practicable after September 1 and no later than January 1. Those not previously immunized should receive, if feasible, a second dose of 1.0 ml. approximately two months after the first injection.

(*2*) *Children six to twelve years of age:* 0.5 ml. (250 CCA units) should be administered subcutaneously as soon as practicable after September 1 and no later than January 1. Those not previously immunized should receive, if feasible, a second dose of 0.5 ml. approximately two months after the first injection.

(*3*) *Children three months old to pre-school age:* Initial doses of 0.1 to 0.2 ml. (50 to 100 CCA units) should be administered subcutaneously on two occasions, separated by one- or two-week intervals. A booster inoculation of the same strength should be given, if feasible, two to three months later. Preferably, the schedule of vaccination should be completed by January 1. Since febrile reactions to vaccine in this age group may reach an incidence of 20 per cent, it is suggested when not contraindicated that acetylsalicylic acid (one grain per year of age) be given every six hours for the first twenty-four hours.

(*4*) *Persons previously immunized with polyvalent vaccine:* Each fall, prior to January 1, persons previously immunized with polyvalent influenza virus vaccine should be reinoculated with a single dose according to the following schedule:

Children three months old to pre-school age: 0.1 to 0.2 ml.

Children aged six to twelve years: 0.5 ml.

Adults: 1 ml.

[1] George Burch, Fred Davenport, Dorland J. Davis, Morris Schaeffer, Glenn S. Usher, and Colin MacLeod, *Chairman*.

Notes

TUBERCULOSIS CASE FINDING AMONG HOMELESS MEN IN NEW YORK CITY

A recently instituted centralized tuberculosis case-finding program for homeless men in New York City offered the writers an opportunity for determining the prevalence of new cases in this susceptible segment of the population. The program utilized the Men's Shelter of the Department of Welfare as a center of operation. In 1960, this facility processed 17,206 "Skid Row" residents.

A 70-mm. photo-roentgen unit, permanently installed at the Shelter, was operated by Health Department personnel from 9:00 a.m. to 5:00 p.m. weekdays. Because of the transient nature of the population, more than 8,000 of the men who received service during the off hours were no longer available to have photo-roentgenograms taken later on. Sputum specimens for culture were collected tri-monthly from residents considered to have inactive[1] pulmonary tuberculosis on the basis of history and roentgenographic findings. All men coming to the Shelter were required to have a photo-roentgenogram taken at least once a year; those with inactive disease had chest films taken every three months as long as they remained under the supervision of the Shelter. All photo-roentgenograms were read twice; the final interpretation was made by the senior writer, with knowledge of each man's previous history of tuberculosis as available from the Central Registration File of the Bureau of Tuberculosis.

The results of the present case-finding program during 1960 are given in table 1. Approximately 9,000 men had at least one chest photo-roentgenogram taken during the year. In 120 instances, the chest photo-roentgenographic findings were considered sufficiently characteristic to warrant an immediate working diagnosis of active pulmonary tuberculosis and prompt referral to a hospital. With only a rare exception, this diagnosis was eventually confirmed. In the other 24 cases, roentgenographic findings of tuberculosis of undetermined activity led to the submission of a sputum specimen for culture and the eventual isolation of tubercle bacilli. Thus, during 1960, a total of 144 new cases of active pulmonary tuberculosis was discovered among 9,000 homeless men, giving a phenomenally high rate of 16.0 per 1,000 clients examined.

[1] In this report, the term "inactive" is used to include all forms of noninfectious pulmonary tuberculosis, with or without discernible cavitation.

During 1960, the present case-finding program among homeless men also uncovered 107 patients with previously known and registered active tuberculosis, almost all of whom had left a hospital against medical advice. An additional 56 patients, among 887 who were followed for "inactive" pulmonary tuberculosis, were found to have reactivation of their disease. Thus, a total of 307 patients with active pulmonary tuberculosis was identified among the 9,000 homeless males during 1960.

All persons diagnosed as having active pulmonary tuberculosis were promptly referred to a hospital. About 20 per cent disappeared before being admitted to the hospital, and another 30 per cent left the hospital against advice within four months. On the other hand, looking at the same statistics optimistically, it can be said that 50 per cent of the men referred to a hospital remained there for at least four months.

* * *

Obviously, tuberculosis continues to be a devastating problem among homeless males in New York City. Present tuberculosis control measures are probably having some effect in controlling the spread of tuberculosis in this group, but they fall far short of what is needed to eradicate the disease. A definitive tuberculosis control program for "Skid Row" residents must include increased and improved facilities for detaining such patients in the hospital, more extensive facilities for rehabilitation after hospital discharge and, most important, a better-coordinated plan for comprehensive medical care to include the treatment of chronic alcoholism.

In summary: Of 9,000 homeless men examined in New York City during 1960, 144, or 16.0 per

TABLE 1

NUMBERS AND RATES OF CASES OF ACTIVE PULMONARY TUBERCULOSIS NEWLY DISCOVERED AMONG APPROXIMATELY 9,000 HOMELESS MEN IN NEW YORK CITY, JANUARY 1 TO DECEMBER 31, 1960

Method of Detection	Number	Rate per 1,000
Roentgenogram only	120	13.3
Roentgenogram and sputum	24	2.7
Total	144	16.0

1,000, were found to have previously undiagnosed active pulmonary tuberculosis.

[2] The writers wish to acknowledge the technical assistance of Rebecca Mencher of the Bureau of Tuberculosis, and the cooperation of the supervisory staff of the Men's Shelter, Department of Welfare, and the Tuberculosis Hospital Facilities of the Department of Hospitals, New York.

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THERAPEUTIC EFFECT OF ETHAMBUTOL (DEXTRO-2,2′-[ETHYLENEDIIMINO]-DI-1-BUTANOL) ON EXPERIMENTAL TUBERCULOSIS IN GUINEA PIGS[1, 2]

The racemic compound of 2,2′-(ethylenediimino)-di-1-butanol was found to have a tuberculostatic effect in mice infected intravenously with *Mycobacterium bovis* or *M. tuberculosis* (H37Rv) when treatment was started on the day of infection and the agent was administered parenterally or orally by intubation or mixture with the diet.[3] It was later shown that the activity of the racemic compound is due to the dextrorotatory form. Dextro-2,2′-(ethylenediimino)-di-1-butanol, or ethambutol, was found to be effective as an antituberculosis agent in mice when treatment was delayed.[4] The following report describes an experiment in which it was found that the administration of the racemic compound to tuberculous guinea pigs had a pronounced therapeutic effect.

Each of 36 male guinea pigs, each weighing approximately 500 gm., was infected intraperitoneally with 0.1 mg. (moist weight) of *M. tuberculosis* strain H37Rv. On the twenty-first day of infection, 6 animals were killed and were found to have tuberculous lesions; there were enlargement of the spleen, multiple miliary lesions in the liver, fibrocaseous masses in the omentum, and enlargement and necrosis of the hepatic and iliac lymph nodes.

On the twenty-first day of infection, the remaining 30 guinea pigs were divided into three groups of 10 animals each, and treatment was started as follows: *group 1* (controls) received 1 ml. of an 0.85 per cent solution of sodium chloride subcutaneously; *group 2* received orally by intubation 50 mg. of the racemic compound in 1 ml. of aqueous solution; *group 3* received by subcutaneous injection 50 mg. of the racemic compound in 1 ml. of aqueous solution. The dose is equivalent to 25 mg. of ethambutol (dextrorotatory form). The treatments were given once daily; the subcutaneous injections were made in the axillary space.

After sixty-two days of treatment, six of the 10 control animals were dead and 3 of the 4 survivors in this group were moribund. The experiment was then discontinued and all surviving animals were killed.

Survival: Referring to figure 1, it will be seen that half of the control animals were dead by the sixty-first day of infection, at which time only 2 in the group treated orally had died, and none in the group treated subcutaneously had died. At this time all but one of the control animals appeared ill and all apparently were losing weight; 2 of the animals treated orally and none of those in the group treated subcutaneously appeared ill. Subsequently, there were a few deaths in the treated groups, as indicated in figure 1, but none of these animals had sufficient tuberculous disease to account for their deaths; one had lesions of pneumonia and 2 had massive intra-abdominal hemorrhage.

Observations at necropsy: There was no necrosis, fibrosis, or hemorrhage in the subcutaneous tissue at the sites of the daily injections of the drug. The grossly visible tuberculous lesions in the lungs, liver, and spleen are shown schematically in figure 1. In the control animals there was extensive disease in the usual organs of predilection, as depicted, plus caseous necrosis of the lymph nodes and fibrocaseous masses in the omentum. It is of interest that the animals treated orally had relatively little pulmonary involvement as compared with the control animals, but they did have extensive involvement of lymph nodes and omentum, and 6 had diffuse involvement of the liver or spleen, or both. In contrast, the visible lesions in the animals which received the drug subcutaneously were limited to miliary or nodular lesions of the liver or spleen in 6 animals; however, all of the guinea pigs in this group had residual caseous foci in the omentum and lymph nodes.

Microscopic examination: The histopathologic appearance of tissues confirmed the observations made grossly. In the control animals there was the progressive, destructive disease in the lungs, liver, and spleen which is typical of experimental tuberculosis in guinea pigs. Among the animals treated orally, 6 exhibited a similar morbid change in the liver and spleen: 3 of these animals had received the full sixty-two days of treatment. However, only 2 in this group had pulmonary lesions comparable in extent to those seen in the control animals; the others had small collections of epithelioid cells with minimal necrosis.

In the animals treated subcutaneously there was

[1] This investigation was supported in part by Research Grant No. E-2357 from the Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service.

[2] The ethambutol was furnished by Dr. Virgil Place, Lederle Laboratories, Pearl River, New York.

[3] Thomas, J. P., *et al.*: Amer Rev Resp Dis, 1961, *83*, 891.

[4] Wilkinson, R. G., *et al.*: J Amer Chem Soc, 1961, *83*, 2212.

much less active tuberculous change as compared with this change in the other two groups. No animal had more than small circumscribed foci of epithelioid cells without necrosis in the lungs. There were scattered and circumscribed lesions in the livers and spleens of all animals, but in only 4 were there necrotic foci, and these were well limited. An index of infection, based on the extent and microscopic character of the lesions, was determined for each group as follows: *group 1* (controls), 73; *group 2* (treated orally), 42; and *group 3* (treated subcutaneously), 15. By this method the presence of active lesions with necrosis was rated from 10 to 35, depending on the extent of involvement; regressive lesions with no necrosis were rated from 1 to 3.[5]

Bacteriologic cultures: Cultures from 6 of the orally treated animals and from 6 of the subcutaneously treated animals were tested for susceptibility to ethambutol in egg-yolk agar in comparison with the susceptibility of the original culture. The cultures were inhibited by 0.5 or 1.0 γ of the agent per ml., as was the control culture, indicating no evidence of increased resistance to the drug.

Toxicity: No attempt was made to study any toxic effects of the treatment, but certain observations suggest that, in the doses used, this material is relatively nontoxic in guinea pigs. No evidence of local irritation such as necrosis, hemorrhage, or fibrosis was seen in the subcutaneous areas at the site of the daily injections. The animals which responded favorably to the tuberculostatic effects remained healthy in appearance, and apparently did not lose weight. The histopathologic studies disclosed no changes that could be attributed to toxicity of the drug. Some of the animals in the treated groups did have a few renal lesions resembling interstitial nephritis, but these changes were seen in the control animals.

* * *

Evidence is available to show that only the dextrorotatory form of 2,2'-(ethylenediimino)-d-1-butanol (ethambutol) is tuberculostatic.[4] Therefore, the actual dose of ethambutol as used in this experiment is half that of the racemic compound, or 25 mg. administered once daily. It is evident that this amount is therapeutically effective when injected subcutaneously once daily.

Assays of the *in vitro* tuberculostatic effect of plasma from treated guinea pigs which had received 50 mg. of ethambutol per kg. of body weight indicate that a maximal concentration of the agent in the blood is reached within one hour to two hours, and that it decreases rapidly so that none is detected after six hours. Additional studies are needed to determine the optimal dose as compared with the amount selected arbitrarily in the present studies. There is now no evidence that any antituberculous drug is more effective against experimental tuberculosis when administered in multiple daily doses than when administered in a single dose.[6]

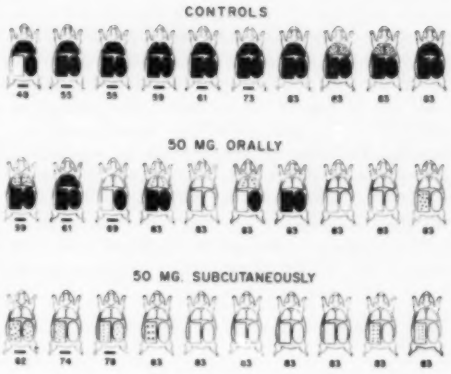

FIG. 1. Extent of the tuberculous lesions seen at necropsy. The rectangle and oval represent the liver and spleen, respectively. Miliary and nodular lesions are indicated by small and large dots; blackening of the organ indicates diffuse involvement. The numerals show the number of days of infection, and a bar below the animal indicates that it died before the end of the experiment. Treatment was given once daily, and was started twenty-one days after infection. Treatment continued for sixty-two days, after which all survivors were killed.

Antibacterial drugs cannot properly be compared on a basis of weight; they must be compared on the basis of therapeutic activity and toxicity. The result achieved by ethambutol against experimental tuberculosis in guinea pigs as described herein is inferior to that expected of a single daily dose of 6 mg. of streptomycin[7] or a single daily dose of 10 mg. of isoniazid.[8] However, if additional studies reveal that the toxicity of ethambutol is low, it may prove to be a valuable antituberculous drug administered alone or in combination with other agents.

In summary: Three groups of 10 guinea pigs each were infected with *Mycobacterium tuber-*

[5] FELDMAN, W. H.: Amer Rev Tuberc, 1943, *48*, 248.

[6] CARR, D. T., AND KARLSON, A. G.: Amer Rev Resp Dis, 1961, *84*, 90.

[7] KARLSON, A. G., AND FELDMAN, W. H.: Amer Rev Tubere, 1948, *58*, 129.

[8] KARLSON, A. G., AND FELDMAN, W. H.: Amer Rev Tubere, 1953, *68*, 75.

culosis and, on the twenty-first day of infection, treatment of the animals in the respective groups was started as follows: (*1*) control animals, 1.0 ml. of an 0.85 per cent solution of sodium chloride; (*2*) 50 mg. of the racemic compound given orally by tube; and (*3*) 50 mg. injected subcutaneously. The dose is equivalent to 25 mg. of the dextrorotatory compound, ethambutol. The treatments were administered once daily in 1 ml. of solution. After sixty-two days of treatment, 6 control animals were dead as compared with 3 in each of the treated groups. Gross and microscopic comparison showed that the treated animals had less disease than the untreated animals. The regressive changes and evidence of healing of the lesions were more pronounced in the animals treated subcutaneously.

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August 3, 1961

THE IN VITRO ACTIVITY OF ETHAMBUTOL (DEXTRO-2,2′-[ETHYLENEDIIMINO]-DI-1-BUTANOL) AGAINST TUBERCLE BACILLI AND OTHER MICROORGANISMS[1]

Ethambutol is the dextrorotatory isomer of 2,2′-(ethylenediimino)-di-1-butanol; it is a white crystalline powder, soluble in water and heat stable. It was found that the racemic compound inhibited the growth of the H37Rv strain of *Mycobacterium tuberculosis* in concentrations of 1 to 4 γ per ml. of Sauton medium.[2] Subsequently, it was shown that the tuberculostatic effect of the racemic compound *in vitro* and in tuberculous mice was caused by the dextrorotatory isomer, ethambutol.[2, 3] The following report presents the method used and the results of determining the *in vitro* activity of this compound against tubercle bacilli, other acid-fast bacteria, and some nonacid-fast forms. It was found that in egg-yolk agar the growth of most strains of human and bovine tubercle bacilli was inhibited by the addition of 1 γ of ethambutol per ml. In the case of other acid-fast bacilli there was a greater variation in susceptibility to the drug; the growth of nonacid-fast bacteria was not inhibited by concentrations of the agent up to 200 times that required to inhibit the growth of *M. tuberculosis*.

Egg-yolk agar containing ethambutol[4] was prepared in the following manner: The ethambutol was taken from its original container with a sterile spatula, weighed in a sterile test tube, and dissolved in sterile Proskauer and Beck liquid medium at a concentration of 10 mg. per ml. This solution was not further sterilized; it was frozen and stored in amounts of 3 ml. For use, the solution was melted at room temperature and diluted in sterile Proskauer and Beck liquid medium to concentrations of 1,000, 500, and 100 γ per ml., respectively. One milliliter of each of these dilutions was added to 90 ml. of melted and cooled sterile trypticase-soy agar (BBL[5]). The yolk from a fresh egg was then added, which made a final volume of egg-yolk agar of approximately 100 ml., with concentrations of 10.0, 5.0, and 1.0 γ, respectively, of ethambutol per ml. The agar was poured into sterile, screw-capped tubes in amounts of 3 to 4 ml., and was slanted.[6] It was used within twenty-four to forty-eight hours.

TABLE 1

COMPARISON OF THE IN VITRO SUSCEPTIBILITY OF VARIOUS MICROORGANISMS TO ETHAMBUTOL

Microorganism	Number Tested	Number of Each Strain Inhibited by Lowest Concentration (γ per Ml.) of Egg-Yolk Agar			
		1.0	5.0	10.0	Not Inhibited by 10.0
M. tuberculosis	170*	115	55		
M. bovis	23	18	5		
M. avium	21		10	3	8
M. ulcerans	3				3
M. fortuitum	14				14
Photochromogens	10	2	8		
Nonchromogens	61	12	24	16	9
Scotochromogens	35	5	21	4	5
Staph. aureus†	140				140
Enterococci	30				30
Coli-aerogenes	67				67
Proteus spp.	22				22
Pseudomonas spp.	28				28

* Includes 4 isoniazid-resistant, 4 PAS-resistant, 3 PAS-and isoniazid-resistant, and 2 PAS- and streptomycin-resistant strains.

† In addition, the following were tested and found to be resistant to more than 100 γ of ethambutol per ml. in trypticase-soy agar: 65 strains of *Staph. aureus*, 34 of enterococci, 52 of coli-aerogenes, 17 of *Proteus spp.*, and 25 of *Pseudomonas spp.*

In the case of acid-fast bacilli, the inoculum was prepared by triturating colonies of the culture in sterile nutrient broth (Difco) in the bottom of a sterile tube by means of a glass rod or an inoculating loop. A final density of approximately five times that of McFarland's nephelometer tube No. 1 was used. Each tube of medium, including a control tube of egg-yolk agar, was inoculated with approximately 0.1 ml. of bacterial suspension. Preliminary observations indicated that the density of the bacterial suspension used for inoculum was not a critical factor, and that uniform results could be obtained when the density was determined by visual inspection alone. Results of the test on acid-fast bacteria were recorded after fourteen days of incubation at 37°C.,

[1] This investigation was supported in part by Research Grant E-2357 from the Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service.

[2] WILKINSON, R. G., *et al.*: *J Amer Chem Soc*, 1961, *83*, 2212.

[3] THOMAS, J. P., *et al.*: Amer Rev Resp Dis, 1961, *83*, 891.

[4] The ethambutol was furnished by Dr. Virgil Place, Lederle Laboratories, Pearl River, New York.

[5] Baltimore Biological Laboratories.

[6] KARLSON, A. G., AND NEEDHAM, G. M.: Proc Mayo Clin, 1948, *23*, 401.

except for *M. ulcerans*, which was incubated at 31°C. for three weeks.

Tests for the susceptibility of the nonacid-fast bacteria were done by placing a drop of six-hour broth culture on the surface of trypticase-soy agar (BBL) containing appropriate concentrations of ethambutol. The results of these tests were recorded after twenty-four hours of incubation.

The data in table 1 provide a comparison of the *in vitro* susceptibility of various kinds of bacteria, and also show the range of susceptibility of strains within a group of organisms. It is of interest to note that the mammalian tubercle bacilli, that is, *M. tuberculosis* and *M. bovis*, appear to be uniformly susceptible to the agent within a relatively narrow range. Moreover, of the 170 strains of *M. tuberculosis*, the growth of 115, or 76 per cent, was inhibited by 1 γ of ethambutol per ml., and growth of the remainder was inhibited by 5.0 γ, the next highest concentration. Inspection of the table reveals susceptibility of the photochromogens comparable to that of the tubercle bacilli. However, the nonchromogens varied widely: 12, or 20 per cent, of the 61 nonchromogens were inhibited by 1.0 γ of the agent and 9, or 15 per cent, were able to grow in the presence of 10 γ per ml. It is not known if the nonchromogens or the scotochromogens, which also varied in susceptibility, are homogeneous groups; these categories may contain different kinds of acid-fast bacteria. However, a wide range of susceptibility was found in the 21 strains of *M. avium*, each of which had been identified by animal pathogenicity tests. The lack of activity of ethambutol against the gram-positive cocci and the gram-negative bacteria is reminiscent of isoniazid and para-aminosalicylic acid, both of which have selective activity for acid-fast bacteria, but fail to inhibit the growth of nonacid-fast bacteria.

In summary: Ethambutol, dextro-2,2′-(ethylenediimino)-di-1-butanol, was tested for inhibitory effect on 170 strains of *Mycobacterium tuberculosis* in concentrations of 1.0, 5.0, and 10.0 γ per ml. of egg-yolk agar. It was found that 115 (76 per cent) of the strains were inhibited by 1.0 γ, and that 55 (24 per cent) were inhibited by 5.0 γ. Twenty-three strains of *M. bovis* and 10 photochromogens exhibited a range of susceptibility comparable to that of *M. tuberculosis*. The *in vitro* susceptibility of *M. avium* (21 strains), nonchromogens (61 strains), and scotochromogens (35 strains) to ethambutol was variable; some strains in each group were inhibited by 1.0 γ per ml., whereas others were not inhibited by 10.0 γ per ml. Ethambutol exerted no inhibitory effect on *Staphylococcus aureus*, *Proteus spp.*, *Pseudomonas spp.*, enterococci, or the coli-aerogenes group.

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PREPARATION OF A HIGHLY POTENT HEMAGGLUTINATING ANTIGEN OF M. TUBERCULOSIS

Although most of the investigators who have been engaged in the elucidation of the nature of the hemagglutinating antigen contained in the culture filtrate or in the cells of *M. tuberculosis* appear to hold the view that the polysaccharide fraction has the activity,[1-5] dissenting opinions still exist.[6-8] Furthermore, the precise chemical nature of the active substance is not yet known.

In the course of the writers' studies on the nature of the antigen, a new simple method was found which made it possible to prepare a more potent antigen than those described to date. The method of preparation, together with some of the chemical properties, will be given in the present report.

The potency of the antigen preparation was expressed by the minimal sensitizing dose. A routine method of sensitization was used: One milliliter of a 4 per cent suspension of sheep erythrocytes was mixed with an equal amount of serial dilutions of the various fractions to be tested, and was incubated at 37°C. for two hours. After washing twice with buffered saline (pH 7.2), the sensitized erythrocytes were taken up into 4 volumes of buffered saline. To 0.2 ml. of this 0.5 per cent suspension of sensitized erythrocytes an equal volume of antiserum dilutions ranging from 1:40 to 1:1,280 was added. The antiserum was prepared by injections of heat-killed bacilli into rabbits. The mixture was incubated at 37°C. for two hours. The minimal sensitizing dose of a fraction was defined as the minimal concentration (γ per ml.) of the fraction required to give a definite agglutination of erythrocytes.

The preparation of a highly potent fraction was made as follows: eight-week-old cultures of *M. tuberculosis* var. *hominis*, strain Aoyama B, grown on Sauton medium were sterilized by heating at 100°C. for one hour, filtered through paper on a Buchner funnel, and then subjected to Seitz filtration. The starting material used for the preparation of the hemagglutinating antigen was the concentrates obtained by ultrafiltration of this culture filtrate. The minimal sensitizing dose of this material was 50 γ per ml., as illustrated in table 1.

To the solution of this starting material, trichloracetic acid was added in a concentration of 5 per cent. The precipitated protein fractions were removed by centrifugation. The supernatant containing the active material was dialyzed and then fractionated by precipitation with ethanol. The fractions precipitated at the ethanol concentration between 40 and 80 per cent were collected by centrifugation and dialyzed against running water. A part of this dialysate was freeze-dried and tested for its activity. The potency of this ethanol-fractionated material was found to be 12.5 γ per ml. A chloroform–amyl alcohol mixture (4:1) was added to the remaining dialysate. The gel formed by vigorous shaking of this mixture was separated by centrifugation, and a fresh chloroform–amyl alcohol mixture was added to the water layer, and then shaken. This procedure was repeated until the amount of gel was reduced so as to form only a thin membrane-like disk at the interface. By this procedure, most of the activity passed into the gel, leaving only a trace, if any, in the supernatant, which failed to sensitize erythrocytes at the concentration of as much as 1,000 γ per ml.

The active substance contained in the gel was precipitated by the addition of 10 volumes of ethanol, dissolved in a small amount of distilled water, and then dialyzed. This procedure was repeated an additional two times so as to remove as many inactive substances contaminated in the gel as possible. The solution of active antigen thus obtained was further treated with trichloracetic acid. The inactive precipitates formed by the addition of this protein precipitant to a concentration of 5 per cent were removed by centrifugation. The sensitizing activity of this supernatant increased to a high degree. One-half gamma per milliliter of this material gave a positive result. The inactive substances still present in the solution were finally precipitated by half saturation with ammonium sulfate, and removed by centrifugation. A highly potent antigen was obtained by lyophilization after dialysis of the supernatant, which was able to sensitize erythrocytes at a concentration of 0.25 γ per ml.

It was found, however, that this material was not homogeneous on electrophoretic examination, and contained inactive polysaccharides and proteins. The material was then further fractionated by zone electrophoresis. The technique described by Kunkel[9] was followed with slight modifications.

[1] Middlebrook, G., and Dubos, R. J.: J Exp Med, 1948, *88*, 521.

[2] Takeda, Y., *et al.*: Jap J Tuberc, 1954, *2*, 307.

[3] Sorkin, E., *et al.*: Helv Chim Acta, 1956, *39*, 1684.

[4] Boyden, S. V., and Sorkin, E.: In *Ciba Foundation Symposium on Experimental Tuberculosis*, edited by G. E. W. Wolstenholme and M. P. Cameron, J. & A. Churchill, London, 1955, p. 144.

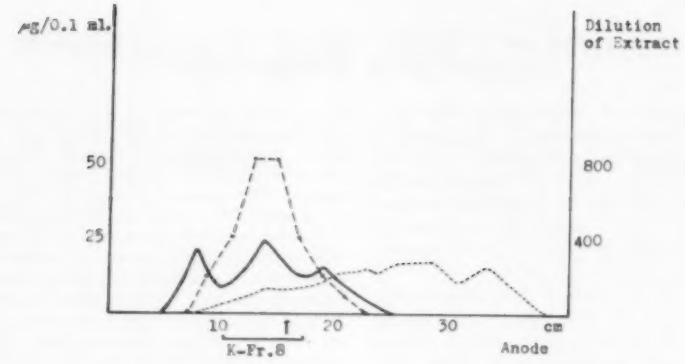
[5] Tsumita, T., *et al.*: Jap J Med Sci Biol, 1959, *12*, 167.

[6] Gericke, D.: Zbl Bakt, 1952, *158*, 191.

[7] Sindo, T., *et al.*: Jap J Exp Med, 1952, *22*, 97.

[8] Iland, C. N., and Peacock, D. B.: In *Ciba Foundation Symposium on Experimental Tuberculosis*, edited by G. E. W. Wolstenholme and M. P. Cameron, J. & A. Churchill, London, 1955, p. 163.

[9] Kunkel, H. G.: In Methods of Biochemical Analysis, Vol. 1, edited by D. Glick, Interscience Publishers, New York, 1954, p. 141.



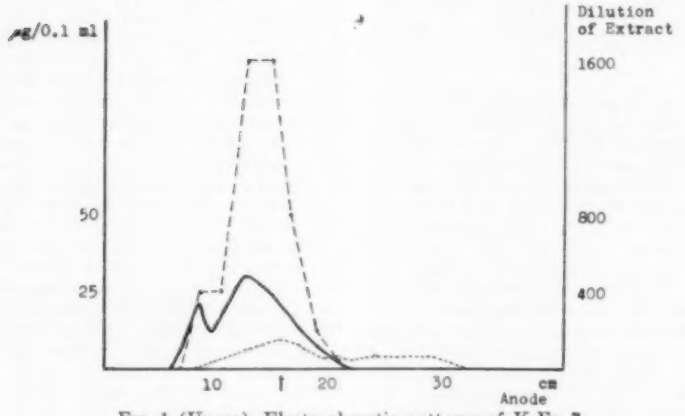


FIG. 1 (Upper). Electrophoretic patterns of K-Fr. 7.
FIG. 2 (Lower). Electrophoretic patterns of K-Fr. 8.
Electrophoresis was done under the following conditions: Starch supporting media, borax-NaH_2PO_4 buffer, pH 8.4, μ 0.05, at 250 to 300 volts, and 2 mA/cm.^{2} for 20 hours in a cold room.
Solid line: Carbazole reaction.
Dotted line: Folin-Ciocalteu phenol reaction (Lowry's modification).
Broken line: Hemagglutinating activities of the extracts.

Borax-monosodium acid phosphate buffer[10] at pH 8.4 and ionic strength 0.05 served as the suspending solution for the starch. Electrophoresis was performed in a cold room at 250 to 300 volts and 2 mA/cm.^{2} for about twenty hours. When electrophoresis was completed, the starch block was divided into sections 1-cm. wide, and each section was suspended and extracted with physiologic saline.

The individual extracts were then analyzed quantitatively for sensitizing activity and for protein and sugar. The protein content was measured colorimetrically by the Folin-Ciocalteu phenol reaction,[11] and the carbohydrate content was determined by the carbazole method. The electrophoretic pattern of this fraction may be seen in figure 1, in which 3 polysaccharide zones and one protein zone are illustrated. The activity was associated with the middle polysaccharide peak, which migrated a small distance toward the cathode. The active polysaccharide fraction recovered from this zone showed an even more elevated activity. This material was re-examined for homogeneity by applying it to a second zone electrophoresis under similar conditions as used previously; the result is illustrated in figure 2. It was found, however, that this material was still nonhomogeneous electrophoretically, and was contaminated with protein and inactive polysaccharides. The sensitizing activity was found in association with the major polysaccharide peak. This material was also examined by ultracentrifugal analysis, and revealed

[10] GEDIN, H. I., AND PORATH, J.: Biochim Biophys Acta, 1957, *26*, 159.
[11] Lowry's modification.

TABLE 1

YIELDS AND HEMAGGLUTINATING ACTIVITIES OF FRACTIONS

Fractionation	Fractions	Yield		Minimal Sensitizing Dose	Hemagglutination Inhibition Dose
		Mg.	Per Cent		
				γ per ml.	*γ per ml.*
K-OT	K-OT	44,000		50	100
K-polysaccharide-80	K-80	5,750	13.0	12.5	10
-S	K-Fr.1	4,852	11.0	1,000	10
-S	K-Fr.2	223	0.5	400	10
G | S	K-Fr.3	60	0.14	10	10
G | -TP	K-Fr.4	52	0.12	100	100
-G | -TS	K-Fr.5	131	0.3	0.5	10
-AP	K-Fr.6	27	0.06	10	
-AS	K-Fr.7	97	0.22	0.25	

K: Lot of tuberculin. K-polysaccharide-80: Precipitates at the ethanol concentration between 40 and 80 per cent from the deproteinized OT. S: Centrifugal supernatant of Sevag's method. G: Chloroform gel. TP: Precipitates at 5 per cent trichloracetic acid. TS: Supernatant at 5 per cent trichloracetic acid. AP: Precipitates at a half saturation with ammonium sulfate. AS: Supernatant at a half saturation with ammonium sulfate.

the presence of 1.9 S fraction as a single component under the conditions examined. This peak showed a slight asymmetry.

As may be seen in table 2, the chemical analysis of the material gave the following results: nitrogen, 3.9 per cent (Dumas method); phosphorus, 1.1 per cent (Allen method); pentose, 15 per cent (cysteine–sulfuric acid method); hexose, 25 per cent (anthron method[12]); and protein, 27 per cent. Evidence for the presence of deoxyribonucleic acid was not obtained. Hydrolysis of this material with 6N hydrochloric acid for nine hours, followed by extraction with ether, gave approximately 20 per cent of an ether-soluble lipid. Analysis of this material by means of reverse-phase chromatography, using the technique described by Nowotony and co-workers,[13] revealed an apparent presence of fatty acids with Rf corresponding to C_{16} and C_{18} in the material. The presence of lipids in the antigen has also been reported by several workers.[5, 6, 14] Mannose and arabinose were demonstrated chromatographically as the sugar components.

This preparation showed a marked activity: 0.05 to 0.1 γ per ml. of the antigen was capable of sensitizing erythrocytes to cause agglutination. However, the antigen had no antigenicity *in vivo*. Three intravenous injections of 1 mg. each into rabbits failed to produce any detectable amounts of antibody.

As stated above, the final product still contained appreciable amounts of protein. Tryptic digestion of the antigen did not have any detectable influence on the sensitizing activity. Together with the above result, this fact seems to support the con-

TABLE 2

CHEMICAL AND BIOLOGIC PROPERTIES OF THE HEMAGGLUTINATING ANTIGEN

Chemical properties	Nitrogen %	3.9
	Phosphorus %	1.1
	Protein %	27.0
	Pentose %	18.0
	Hexose %	25.0
	Deoxyribonucleic acid %	0
	Ether-soluble substance	about 20%, C_{16}, C_{18} fatty acid
	Constituent sugars	mannose, arabinose
Biologic properties	MSD* Hemagglutination	0.05 γ per ml.
	MSD* Hemolytic modification†	0.01 γ per ml.
	Precipitin titer‡	1:200,000
	Antigenicity§	—

* MSD: Minimal sensitizing dose.

† Complement: 2 exact units.

‡ Antiserum prepared by intravenous injection of heat-killed bacilli.

§ 3 Intravenous injections, 1 mg. each, into rabbits.

[12] MORRIS, D. L.: Science, 1948, *107*, 254.

[13] NOWOTONY, A., *et al.*: Biochem Z, 1958, *330*, 47.

[14] TSUMITA, T., *et al.*: Jap J Med Sci Biol, 1960, *13*, 131.

cept that the active material is a lipopolysaccharide. In addition, there are two facts which suggest the presence of a bound protein moiety. First, this antigen was removed into chloroform gel. Second, the major polysaccharide peak in the electrophoretic pattern was accompanied by a small protein peak. Thus, it would appear that this antigen occurs in culture filtrate as a lipopolysaccharide-protein complex.

In summary: By a combination of the chloroform-gel method and electrophoretic separation, a highly potent hemagglutinating antigen was prepared from the culture filtrate of human tubercle bacilli, strain Aoyama B. The final preparation, which on electrophoretic examination was still nonhomogeneous and contained inactive protein and polysaccharides, was able to sensitize sheep erythrocytes and to cause hemagglutination at a concentration as minute as 0.05 to 0.1 γ per ml. Besides a major polysaccharide component, it produced, on hydrolysis, an ether-soluble lipid substance in amounts approximately 20 per cent of its weight. Tryptic digestion had no influence on the sensitizing capacity. These facts seem to suggest the lipopolysaccharide nature of the antigen.

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April 6, 1961

AN EVALUATION OF THE AURAMINE FLOOD TEST FOR MYCOBACTERIA[1]

Erlich[2] recently found that alkaline auramine reagent flooded on mycobacterial colonies growing on Dubos oleic-agar stained the colonies a brilliant yellow *in situ*. Erlich stated that in his laboratory the auramine test was routinely employed successfully on all cultures before discarding them as negative, and that contaminated plates were similarly tested. A positive auramine flood test would establish the presence of an auramine-positive strain of mycobacteria. A negative test would exclude the presence of *M. tuberculosis*.

The present study was undertaken with the following objectives in view: to determine if any separation of the groups of unclassified acid-fast bacteria occurred when the organisms were grouped according to the Runyon scheme[3]; to observe if a negative auramine test would exclude the presence of *M. tuberculosis*; to find out if many strains of mycobacteria are auramine negative; to see if additional positive cultures, on primary clinical isolation, could be found by gross inspection in the presence of contaminants by use of the auramine flood technique; to compare the standard Löwenstein-Jensen medium, which is not suitable for the flood technique,[4] with the Dubos oleic-agar medium recommended by Erlich,[5] with or without malachite green, for primary isolation.

The strains of mycobacteria used in this investigation were graciously supplied by Dr. E. Runyon,[6] Dr. W. R. Barclay,[6] Dr. T. Toda,[7] Miss S. McMillen,[8] and Mr. H. Erlich.[9]

All strains were subcultured in Dubos Tween®-albumin liquid medium to obtain dispersed growth before being subcultured on Dubos oleic-agar in screw-capped 30-ml. prescription bottles. Cultures were incubated for four to six weeks at 37°C., in the dark, before being tested.

The auramine flood test: Using the method of Erlich,[5] equal volumes of filtered alcoholic auramine solution (0.1 gm. auramine O[10] per 100 ml. of ethyl alcohol) and 1.0 N aqueous sodium hydroxide were mixed just prior to the test. Two to three milliliters of this reagent were then allowed to flood over the colonies. Within one minute the auramine-positive colonies selectively began to extract the dye from the alkaline reagent and stained a brilliant yellow *in situ*, contrasting with the almost colorless background of the culture medium.

This test was interpreted as auramine positive if the yellow stain persisted within the colonies after one minute. By this method, auramine-negative colonies did not visibly extract the auramine dye from the reagent in the presence of alkali and alcohol. Colonies staining yellow but decolorizing within one minute were also designated as auramine negative. The tests were always done against the dull black background provided by a piece of carbon paper, and read by transmitted light.

As a control, Ziehl-Neelsen stains were done on all of the mycobacterial strains studied immediately prior to the auramine flood test.

Media comparison: Routine sodium hydroxide–digested specimens from tuberculous sources were inoculated simultaneously in Dubos oleic-agar medium, in the same medium with 1γ per ml. malachite green, and on the routinely used Löwenstein-Jensen medium. Ziehl-Neelsen and auramine flood techniques were done on cultures after a six-week incubation period.

From the results presented in table 1, it is apparent that only the strains of *M. tuberculosis*, which included laboratory strains such as H37Rv and R1Ra as well as clinical isolates, were uniformly auramine positive. In *Group I*, the photochromogens, 96 per cent of the strains were auramine positive. However, in *Group II*, the scotochromogens, and *Group III*, the Battey strains, a significant number were negative, 21 per cent and 27 per cent, respectively. *Group IV*, the rapid growers, contained more than half of the auramine-negative strains found in all of the groups. In addition, 4 avian strains were found to be auramine positive.

The results of the media comparison, which may be seen in table 2, revealed a very high degree of contamination (more than half of the plate contaminated) on Dubos oleic-agar medium.

[1] The statements and conclusions published by the writers are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

[2] Erlich, H.: Amer Rev Resp Dis, 1960, *81*, 218.

[3] Runyon, E.: Med Clin N Amer, 1959, *43*, 273.

[4] Erlich, H.: Personal communication.

[5] Veterans Administration Hospital, Salt Lake City, Utah.

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[7] Department of Bacteriology, School of Medicine, Kyushu University, Fukuoka, Japan.

[8] Hektoen Institute for Medical Research, Chicago 12, Illinois.

[9] Arroyo Del Valle Sanatorium, Livermore, California.

[10] Manufactured by National Aniline Division of Allied Chemical and Dye Corporation, New York, New York.

TABLE 1

SUMMARY OF AURAMINE FLOOD TEST RESULTS

	M. tuberculosis	Runyon Group				Grand Totals
		I	*II*	*III*	*IV*	
Total	55	48	42	26	36	207
Positive	55	46	33	19	16	169
Negative	0	2	9	7	20	38
Per cent negative	0	4	21	27	56	18

TABLE 2

COMPARISON OF DUBOS OLEIC-AGAR WITH LÖWENSTEIN-JENSEN MEDIUM FOR PRIMARY ISOLATION OF M. TUBERCULOSIS

	Number of Specimens	Cultures Contaminated	Positive Specimens
Dubos oleic-agar	191	39	49
Dubos oleic-agar plus malachite green	191	10	53
Löwenstein-Jensen	191	0	57

This medium showed the lowest number of cultures positive for *M. tuberculosis*.

Intermediate was Dubos oleic-agar medium with malachite green added. Less contamination occurred and more positive specimens were obtained. However, the best results were obtained with standard Löwenstein-Jensen medium, which not only had the highest number of positive cultures, but no contaminations.

All strains were acid fast by the Ziehl-Neelsen technique.

* * *

As stated previously, all of the strains of *M. tuberculosis*, including the avian strains, were auramine fast. Most of the *Group I* strains, the photochromogens, were also auramine positive. By contrast, more than half of the strains tested in *Group IV*, the rapid growers, were negative, while in *Group II*, the scotochromogens, and in *Group III*, the Battey strains, a number of auramine-negative strains were found. This indicates that a clear-cut separation of unclassified mycobacteria from *M. tuberculosis* cannot be made by use of the auramine flood test. Furthermore, groups cannot be clearly differentiated by this technique.

When contaminated cultures of tubercle bacilli from clinical sources (*see* table 2) were examined grossly after the auramine flood test and Ziehl-Neelsen smears were made, no additional acid-fast organisms were demonstrated. Furthermore, the fact that all of the cultures of *M. tuberculosis* tested were auramine positive indicates that a negative auramine flood test excludes the presence of *M. tuberculosis*. However, even if the auramine flood test is negative the culture may still be grossly positive for unclassified mycobacteria, especially *Group IV* strains. Thus, this test is no substitute for Ziehl-Neelsen staining for final identification.

Finally, the media evaluation results, shown in table 2, revealed the standard Löwenstein-Jensen medium to be superior to Dubos oleic-agar medium, either with or without malachite green, based on both the total number of positive cultures and contaminated cultures.

In summary: The auramine flood test is no substitute for the Ziehl-Neelsen stain for the identification of mycobacteria. Standard Löwenstein-Jensen medium is superior to Dubos oleic-agar, with or without malachite green, for the primary isolation of *M. tuberculosis*. No clear separation of the groups of unclassified mycobacteria, according to the Runyon scheme, could be achieved by using the auramine flood test. No additional cultures positive for *M. tuberculosis* from processed clinical specimens could be found by either Ziehl-Neelsen staining or auramine flood testing of contaminated cultures.

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May 31, 1961

[11] The writers gratefully acknowledge the technical assistance of Mr. E. Chalfont.

INHIBITION OF MYCOBACTERIAL CATALASE ACTIVITY BY ISONIAZID

The bacteriostatic activity of isoniazid is apparently specific for mycobacteria,[1, 10] and the mycobacteria resistant to this effect of isoniazid exhibit either lower catalase-specific activity or no catalase activity.[2, 3] Relative to an attempt to determine a phase of the mechanism of the action of isoniazid, a study of mycobaterial catalase was undertaken. The possibility was investigated that isoniazid may have an effect on mycobacterial catalase in cell-free extracts that has not been demonstrable in whole cell preparations.[4]

Preparation of cell-free extracts: As the source of the enzyme catalase, *Mycobacterium smegmatis* was used. The bacterial cells were transferred from a fresh culture onto the surface of 250 ml. of Proskauer and Beck liquid medium in large toxin bottles, incubated at 37°C. for seventy-two hours, and then harvested on the surface of coarse sintered glass filters, removing the medium by suction. The cells were washed with 2 liters of cold (10 to 15°C.) glass-distilled water. This cell mass was weighed and a cell paste prepared in a sterilized mortar with a pestle, using two parts cell mass to one part 0.1M phosphate buffer at pH 6.8 (weight/volume). The paste was placed in a Raytheon 9KC sonic oscillator and treated for fifty-five minutes. Essentially all of the active enzyme was present in the straw-colored supernatant following centrifugation at 15,000 × G for thirty minutes at 0°C. The cell extracts were standardized in respect to the protein content by microkjeldahl and Biuret tests.[5, 6]

Catalase assay and inhibition: The reaction mixture consisted of 0.2 ml. of the standardized extract, 0.2 ml. of isoniazid (0.05, 0.1, and 1M), and 3 ml. of buffered hydrogen peroxide (0.010, 0.021, and 0.031M) in 50-ml. Erlenmeyer flasks. The cell-free extract was preincubated with the isoniazid for fifteen minutes at 37°C. in a shaking water bath before the substrate was added. The reaction was allowed to run sixty seconds and was stopped by blowing 2 ml. of N sulfuric acid into the reaction flask; 0.2 ml. of phosphate buffer was then added. The control assays were performed by first preincubating the extract with the buffer and then adding the buffered substrate, completing the reaction mixture. The reaction was stopped with N sulfuric acid followed by the addition of 0.2 ml. of isoniazid (0.05, 0.1, and 1M). Chemical blanks were established by first adding the sulfuric acid to the preincubated enzyme preparation, and subsequently introducing the substrate solution (with the appropriate incubation period) and the isoniazid solutions. After removal from the water bath, the residual hydrogen peroxide in the reaction flasks was back-titrated *in situ* according to the method of Herbert and Pinsent.[7] It was necessary to keep the flasks stoppered after adding the ammonium molybdate (2 drops of 1 per cent) and the 0.6 ml. of 10 per cent potassium iodide to minimize the loss of iodine by evaporation during the three-minute period of standing before titration with sodium thiosulfate. The titrations were performed with a magnetic stirrer and a burette, fitted at the base with a stopper which was loosely insertible into the mouth of each of the flasks.

A reduction in the specific activity of the mycobacterial catalase by isoniazid at each concentration was demonstrable following fifteen minutes of preincubation at 37°C. (table 1).

Kinetic studies show that isoniazid inhibits the mycobacterial catalase in cell-free extracts in a noncompetitive manner. Lineweaver-Burk plots,[8, 9] least square values of 1/v versus 1/S, show that under these experimental conditions the inactivation of the enzyme is independent of the substrate concentration (figure 1). The data incorporated in these plots are the results of one hundred and eighty-four enzyme assays.

* * *

Isoniazid has been found capable of inhibiting the activity of mycobacterial catalase in cell-free extracts. This finding suggests another facet of the manner in which isoniazid exerts its bacteriostatic effect on mycobacteria. The writer's data are in contrast to those of Knox,[4] who was unable to demonstrate manometrically that isoniazid (0.1M) inhibited the catalase activity of whole cell preparations of BCG strains of mycobacteria and *Staphylococcus aureus* (Oxford strain), as well as crystalline catalase (10^{-5}). The apparent discrepancy may be explained by taking cognizance of the work of Armstrong[10] and that of Koch-Weser and associ-

[1] Pope, H.: Amer Rev Tuberc, 1956, *73*, 735.

[10] Willett, H. P.: Proc Soc Exp Biol Med, 1958, *99*, 177.

[2] Peizer, L. R., and Widelock, D.: Amer Rev Tuberc, 1955, *72*, 246.

[3] Middlebrook, G.: Amer Rev Tuberc, 1954, *69*, 471.

[4] Knox, R., *et al.*: Amer Rev Tuberc, 1956, *73*, 726.

[5] Hiller, A., *et al.*: J Biol Chem, 1948, *176*, 1401.

[6] Weichselbaum, T. E.: Amer J Clin Path, 1946, *7*, 40.

[7] Herbert, D., and Pinsent, J.: Biochem J, 1948, *43*, 193.

[8] Michaelis, L., and Menten, M. L.: Biochem Z, 1913, *49*, 333.

[9] Lineweaver, H., and Burk, D.: J Amer Chem Soc, 1934, *56*, 658.

[10] Armstrong, A. R.: Amer Rev Resp Dis, 1960, *81*, 498.

TABLE 1

ISONIAZID INHIBITION OF MYCOBACTERIAL CATALASE IN CELL-FREE EXTRACTS

Preincubation Isoniazid Concentration	Assay Conditions*	Hydrogen Peroxide Concentration in Reaction Mixture (Mean Values)		
0.0M	S_0 (Blank)	36.89μM	71.47μM	107.73μM
	S_c (Control)	28.91	44.05	51.16
0.05M	S_i	19.12μM	28.26μM	34.18μM
	Difference $= S_c - S_i = \Delta S$	9.79	15.79	16.98
	% Inhibition $= \Delta S/S_c$	33.8%	35.8%	33.1%
0.1M	S_i	18.42μM	26.35μM	32.78μM
	ΔS	10.49	17.70μM	18.38μM
	$\Delta S/S_c$	36.2%	40.1%	35.9%
1.0M	S_i	7.48μM	10.89μM	13.28μM
	ΔS	21.43	33.16	37.88
	$\Delta S/S_c$	74.1%	73.0%	74.0%

* S_0 = reagent blank; reagent sequence as follows: enzyme (E) + buffer (B) + sulfuric acid + peroxide (S) + (1 min. at 37°C.).

S_c = uninhibited assay; sequence: E + B + S + (1 min. at 37°C.) + sulfuric acid + isoniazid (I). The amount of S used.

S_i = inhibited assay; sequence: E + I + S + (1 min. at 37°C.) + sulfuric acid + B. The amount of S used by inhibited enzyme.

ΔS = amount of inhibition: $S_c - S_i = \Delta S$. This much of the enzyme activity was rendered unavailable by the inhibitor.

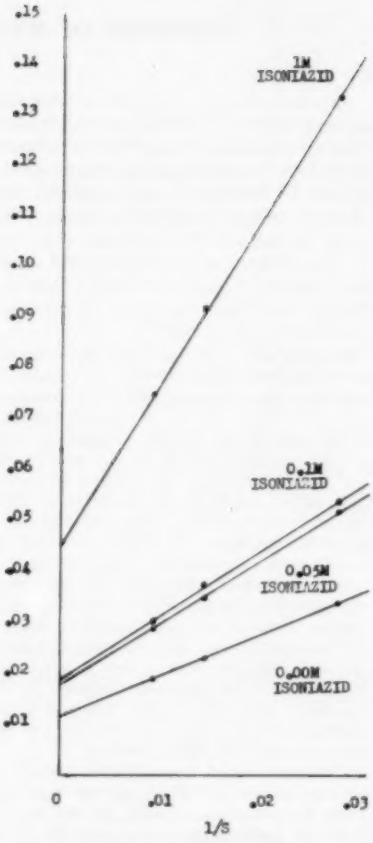

FIG. 1. Inhibition of mycobacterial catalase activity by isoniazid. Lineweaver-Burk plot of 1/v (in μM of hydrogen peroxide utilized min.$^{-1}$) versus 1/S (in μM of hydrogen peroxide).

ates.[11] These investigators have shown with whole cell preparations (of *Mycobacterium tuberculosis* H37Rv) that the bacteriostatic effect of isoniazid is not immediate and, second, that drug-susceptible organisms do adsorb and fix C^{14}-labeled isoniazid to the cell substance. The preincubation of the enzyme and the inhibitor is essential. In addition, the treatment in preparation of the cell-free extracts may obviate the neutralization of isoniazid by a number of metabolites.[1, 10] The relevance of this inhibition to isoniazid chemotherapy is suggested by the fact that the two lowest concentrations of isoniazid employed are within the range of attainable serum concentrations determined by chemical and microbiologic assay.[12] The two drug concentrations elicited essentially the same response. The molar concentration of isoniazid must be considered in respect to the implication of the work of Barclay and coworkers[12] that drug-susceptible tubercle bacilli concentrate radioactive isoniazid, and thus afford an opportunity for reaching a high concentration of isoniazid within the cell.

In summary: Isoniazid in concentrations comparable to those reported in human serum during chemotherapy have been found to inhibit myco-

[11] KOCH-WESER, D., *et al.*: Amer Rev Tubere, 1955, *71*, 556.

[12] POOLE, N. F., AND MEYER, A. E.: Proc Soc Exp Biol Med, 1958, *98*, 375.

[12] BARCLAY, W. R., *et al.*: Amer Rev Tubere, 1953, *67*, 490.

bacterial catalase activity in cell-free extracts in a noncompetitive manner following preincubation for a period of fifteen minutes at 37°C. The experimental evidence presented suggests another facet of isoniazid activity which may contribute to the over-all inhibitory effect of isoniazid.

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June 27, 1961

Correspondence

CERTAIN SAPROPHYTIC MYCOBACTERIA FROM SOIL SOURCES FORMING A RED COLOR PRODUCT FROM PAS

To the Editor of the American Review of Respiratory Diseases:

The present writer has found that one group of unclassified (presumably saprophytic) mycobacteria isolated from soil forms a red color product from PAS (sodium *p*-aminosalicylate). Formation of this color product in egg medium containing 2 mg. of PAS per ml. caused medium that contained malachite green to stain black. This black staining of the medium is presumably derived from a coexistence of the red color of the product and the malachite green (figure 1).

The reddish coloring product was believed to be an oxidation product of catechol.

It was demonstrated that the red formazan did not contain an aromatic amine structure, and it showed the same Rf values (at least three different spots were differentiated) as the red color products from cells plus catechol. All of the extracts obtained from the different spots of cells plus PAS, as well as those obtained from cells plus catechol, showed the same type of absorption spectra, although they showed different Rf values in paper chromatographic studies (solvents: *n*-butanol saturated with water, *n*-butanol saturated with ammonia water, and *n*-butanol-acetic acid (4:1)).

This color product could be produced when the cells of the mycobacteria were incubated at 37°C. for twelve to sixteen hours in M/15 phosphate buffer solution, pH 7.1, containing 2 mg. of PAS per ml. (incubation was done under slow shaking).

The PAS-degrading mycobacteria have been isolated, to date, only from unclassified (presumably saprophytic) mycobacteria from soil sources. Thus far, 11 strains of such mycobacteria, 2 scotochromogens and 9 nonphotochromogens,[1] have been found among 53 strains of unclassified mycobacteria isolated from soil (approximately 20 per cent). All of the strains showed slow growth in Sauton medium. Such mycobacteria were not found among 46 strains of unclassified mycobacteria and 6 strains of saprophytic mycobacteria, of which 37 strains of the former and all of the latter grew abundantly on egg medium containing 2 mg. of PAS per ml. No such mycobacteria were found among 146 strains of *M. tuberculosis*, including PAS-resistant strains. It is suggested, therefore, that unclassified mycobacteria from human sources may contain some strains which are different from those obtained from external environments.

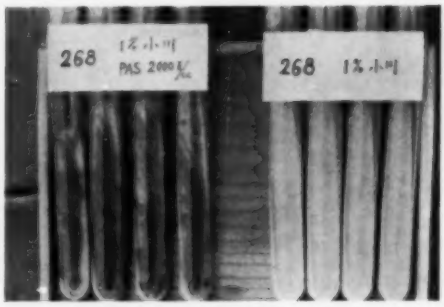

FIG. 1 (Left). Mycobacterium No. 268 growing on Ogawa egg medium containing 2 mg. of PAS per ml. (Right). The same strain growing on Ogawa egg medium containing no drug.

Composition of Ogawa egg medium: basal solution (1 per cent sodium glutamate and 1 per cent potassium dihydrogen phosphate), 100 ml.; whole eggs, 200 ml.; glycerol, 6 ml.; 2 per cent solution of malachite green, 6 ml. Slanted by sterilization at 90°C. for sixty minutes.

In addition, it was noted that 100 per cent of both the saprophytic mycobacteria tested (6 strains) and the unclassified mycobacteria from soil sources (53 strains), and 80 per cent of the unclassified mycobacteria from human sources (46 strains) grew abundantly on egg medium containing 2 mg. of PAS per ml., whereas all of the strains of *M. tuberculosis* tested (146 strains) did not grow, or showed only small numbers of discrete colonies on the medium.

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July 10, 1961

[1] RUNYON, E. H.: Amer Rev Tuberc, 1955, *72*, 866.

Books

HAEMOPOIESIS. CELL PRODUCTION AND ITS REGULATION. Ciba Foundation Symposium. Edited by G. E. W. Wolstenholme and Maeve O'Connor. Pp. 490, Little, Brown and Company, Boston, 1960. Price, $11.00.

This symposium on hemopoiesis, held February 2 to 4, 1960, was attended by twenty-seven investigators of different backgrounds from England, Italy, Denmark, Austria, Sweden, Palestine, Canada, and the United States. Publication was arranged quickly, while the information was still fresh. The book contains sixteen interesting papers, complete lists of recent references, extensive yet pertinent discussion by the participants, and indices according to author as well as subject.

The Chairman of the Conference, J. M. Yoffey, discusses in the keynote paper the evolution of the lymphomyeloid tissues and the distribution, proliferation, migration, destruction, and functions of the derived cells. Two papers, one by N. B. Everett, W. O. Ricke, W. O. Reinhardt, and J. M. Yoffey and the other by E. P. Cronkite, V. P. Bond, T. M. Fliedner, and S. A. Kielmann are concerned with the use of thymidine labeled with the radioisotope tritium in the study of blood cell proliferation and desoxyribonucleic acid replication. G. Astaldi discusses the changes which occur in normal and pathologic bone marrow cells after *in vitro* tissue culture. J. F. Loutit reviews the effects of transfusions of hemopoietic cells on blood cell formation following irradiation. The relationships of thymus lymphocytes to other blood cells are considered in papers by F. Bierring and K. E. Fichtelius. The kinetics of granulocytopoiesis is completely reviewed by C. G. Craddock, M. A. Maloney, and H. M. Patt. Elaborate models as aids to understanding the kinetics of lymphocyte, plasmocyte, and erythrocyte formation are presented by C. P. Leblond, G. Sainte-Marie, L. G. Lajtha, and R. Oliver. The last portion of the volume is devoted to the humoral factors involved in hemopoiesis, and reports are made by M. Rachmilewitz, O. Stein, Y. Stein, E. Simon, J. Gross, A. S. Gordon, J. W. Linman, F. H. Bethell, D. C. Van Dyke, L. O. Jacobson, E. Goldwasser, and C. W. Gurney.

With such a wealth of information on the physiology of blood cell formation, it is interesting though not surprising that there are still problems concerning morphology at the cellular level. How is the plasma cell distinguished from the lymphocyte? How should the different types of lymphocytes be classified? What morphologic cell type is the long-sought-for stem cell? Some workers are familiar with pathologic sections of tissue blocks; others rely upon smears stained in different ways. Still others work with the electron microscope and think in terms of ultrastructure. Very few investigators have directly compared the different techniques and are able to act as translators. The importance of such anatomic considerations is inferred time and again.

The book will appeal for the most part to specialists in hematology. However, the work is so fundamental, touching on many aspects of health and disease, that it may be read with profit by any serious student of disease. The discussion which follows each paper is sufficiently informal so as to be spontaneous. Embarrassing questions are often asked which illuminate dark corners of investigation. Several experiments are proposed which should settle important questions. Editing has been kept to a minimum so that considerable individuality of presentation remains. The format is in keeping with the other books in the series, and has withstood the test of time. As a whole, the avowed purpose of the Symposium as expressed in the preface has been realized, that "students of this book could find in its pages a 'stem cell' totipotent for a rich variety of further research."

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SURGICAL DISEASES OF THE LUNG. By Buford H. Burch and Arthur C. Miller. Pp. 128, Charles C Thomas, Springfield, Illinois, 1961. Price, $8.50.

This monograph consists of ten chapters which review the major aspects of pulmonary surgery. It is written in a clear, precise fashion and achieves the authors' objective to state their attitudes "as briefly and as pointedly as possible." The text is devoted to the practical problems of diagnosis, selection of patients for operation, and choice of surgical procedures in the commonly encountered diseases of the lungs. It represents the authors' personal approach to each disease, and makes no effort to cite other opinions or review controversial methods of management.

Little emphasis is placed on surgical technique with the exception of a considerable discussion of the value and technical aspects of segmental resection. The chapters on tuberculosis and pulmonary tumors are outstanding and summarize the problems, methods of management, and prognosis

in a concise, logical fashion. The chapter on traumatic injuries of the chest is adequate, although some aspects such as flail chest injuries are covered very briefly. The great value of tracheostomy as part of the definitive management of a flail chest is perhaps not sufficiently emphasized.

This is not intended as a reference book or manual and, consequently, the lack of bibliography or references does not detract from its purpose. The greatest asset of this book is its readability, and the succinct presentation of each subject, interspersed with euphemisms, adds greatly to the reader's interest. The sixty roentgenograms in the back of the book are excellent and are classical pictures of the various disease entities discussed in the text.

This book is thoroughly recommended for the house officer and practitioner as a practical and accurate survey of the field of modern pulmonary surgery.

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Symposium on Guanethidine (Ismelin®). The University of Tennessee College of Medicine. Pp. 119, Ciba Pharmaceutical Products, Inc., Summit, N. J., 1960.

Published reports of new antihypertensive drugs—their indications, contraindications, effects, side effects, et cetera—are more numerous than the bones in the Hudson River shad. For this reason, symposia often speed up knowledge of these agents by collecting outstanding workers in the general field and presenting their findings. As long as these presentations are factual and unbiased, they are most worth while.

The Symposium on guanethidine seems to meet these requirements. Nineteen papers describe the chemistry, physiology, pharmacology, and clinical effectiveness of the agent. After reviewing each presentation, the reader is left with the definite impression that this drug is unique in its action, is a powerful antihypertensive, and may be of definite value in severe cases that have not responded to the older measures.

Guanethidine apparently acts primarily by interfering with the metabolism of nerve impulse transmitter material at the nerve terminal. When given orally or by injection, it reduces the blood pressure in hypertensive dogs and humans. Its effect is primarily orthostatic in that, although the supine blood pressure may be reduced to a modest degree, the greatest blood pressure drop occurs when the subject stands erect. It depresses the heart rate and produces an increase in the blood pressure reduction following the Valsalva maneuver, and abolishes the blood pressure overshoot that may occur subsequent to this test. A low degree of tolerance is encountered almost uniformly. When given in moderately high doses, the glomerular filtration rate and renal plasma flow may fall in a majority of cases. As a result, nitrogen retention may take place. An unusual action of the drug is found when a subject on oral doses is given the same material by vein. The parenteral dose may produce a prominent pressure response in many cases.

Physiologic reactions to chemical agents are rarely if ever restricted to the beneficial responses by a single system. Other organs also react, and may do so in ways that are not favorable to the subject or his well being. Guanethidine is no exception to this rule. Its side effects may be severe, with orthostatic weakness and dizziness, which have been encountered by most of the observers in more than half of the patients treated. Diarrhea with occasional abdominal cramps was noted in at least 35 per cent of the patients examined in one study. Although the inability to ejaculate set in with many of the males treated, impotence was not seen to a significant degree. It was interesting that side effects of the medication, produced by parasympathetic inhibition, were not encountered. The major action of the drug appears to be basically on the sympathetic nervous system per se.

At the Symposium, the general consensus was that guanethidine, when used judiciously in the proper patient, was a useful addition to therapeutic drugs for the treatment of severe hypertension. In spite of somewhat more recent opinions that the prevalence and magnitude of side effects interfere almost completely with the use of this drug, the writer's experience in a large number of cases treated over many months is in accord with the general opinion of the papers published in the symposium. As with any therapeutic, guanethidine should be given only when the more simple, less powerful, and less troublesome components of medical management of hypertension have failed to produce the desired result. Other agents used in conjunction with it may often enhance its usefulness.

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The Year Book of Drug Therapy—1960–1961. Edited by Harry Beckman. Pp. 587, The Year Book Publishers, Inc., Chicago, 1961. Price, $8.50.

This book contains abstracts of articles dealing with the drug therapy of diseases and disease states. The summaries are grouped under broad systems or dis-

ease types except when a great deal has been written on one disease, i.e., diabetes mellitus. As such, the volume is a very useful selection of often good articles easily accessible.

As in other *Year Books,* the editor's comments that frequently follow the article are a valuable feature.

Particularly interesting in this volume is the section by Dr. Beckman, called "A Critical Evaluation of the Year's New Drugs," which includes a worth-while essay on "The Bases for Judgment of a New Drug."

For this first section alone, as well as for the review of current literature, this *Year Book* can be recommended as a valuable addition to a general library.

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BOOKS RECEIVED

The Chemotherapy of Tuberculosis. By William F. Russell, Jr., and Gardner Middlebrook. Pp. 130, Charles C Thomas, Springfield, Illinois, 1961. Price, $6.50.

The Nature of Sleep. Ciba Foundation Symposium. Edited by G. E. W. Wolstenholme and Maeve O'Connor. Pp. 416, Little, Brown and Company, Boston. Price, $10.00.

Problems of Pulmonary Circulation. Ciba Foundation Study Group No. 8. Edited by A. V. S. de Reuck and Maeve O'Connor. Pp. 96, Little, Brown and Company, Boston, 1961. Price $2.50.

Thoracic Diseases. Emphasizing Cardiopulmonary Relationships. By Eli H. Rubin and Morris Rubin. Pp. 968, W. B. Saunders Company, Philadelphia and London, 1961. Price, $25.00.

Einführung in die Diagnostik und Begutachtung der Silikotuberkulose. By Gustav Sepke. Pp. 177, Veb Gustav Fischer Verlag, Jena, 1961. Price, 36,60 DM.

Preventive Medicine in World War II. Volume V. Communicable Diseases: Transmitted Through Contact or by Unknown Means. Edited by John Boyd Coates, Jr., Ebbe Curtis Hoff, and Phebe M. Hoff. Pp. 530, Office of the Surgeon General, Department of the Army, Washington, D. C. 1960. Price, $5.75.

American Thoracic Society

Medical Section of the National Tuberculosis Association

NOTICES

The following dates and locations of the next three annual meetings of the National Tuberculosis Association and the American Thoracic Society have been selected and approved:

1962—Miami Beach, Florida—May 20-24
1963—Denver, Colorado—May 12-16
1964—New York, New York—May 24-28

AMERICAN SOCIETY OF CLINICAL RADIOLOGY

The American Society of Clinical Radiology announces that a clinic will be held from January 18 to 20, 1962, at the Arizona Biltmore, Phoenix, Arizona. Chest physicians who do their own diagnostic radiology are invited to attend. For information write Dr. Louis Shattuck Baer, Secretary, 411 Primrose Road, Burlingame, California.

INTERNATIONAL CONFERENCE ON OPPORTUNISTIC (SECONDARY) FUNGAL INFECTIONS

The International Conference on Opportunistic (Secondary) Fungal Infections will be held in Durham, North Carolina, from June 28-30, 1962. For information contact Dr. Ernest W. Chick, Secretary, Veterans Administration Hospital, Durham, North Carolina.

ANNOUNCEMENT—RESEARCH GRANTS

The National Tuberculosis Association, through its medical section, the American Thoracic Society, offers grants for research in tuberculosis and other respiratory diseases. Grants are awarded for medical and social research in these fields. Applications for the grant year beginning July 1, 1962, must be received by December 15, 1961.

Full information and application forms may be obtained from: Division of Research and Statistics, American Thoracic Society, 1790 Broadway, New York 19, New York.

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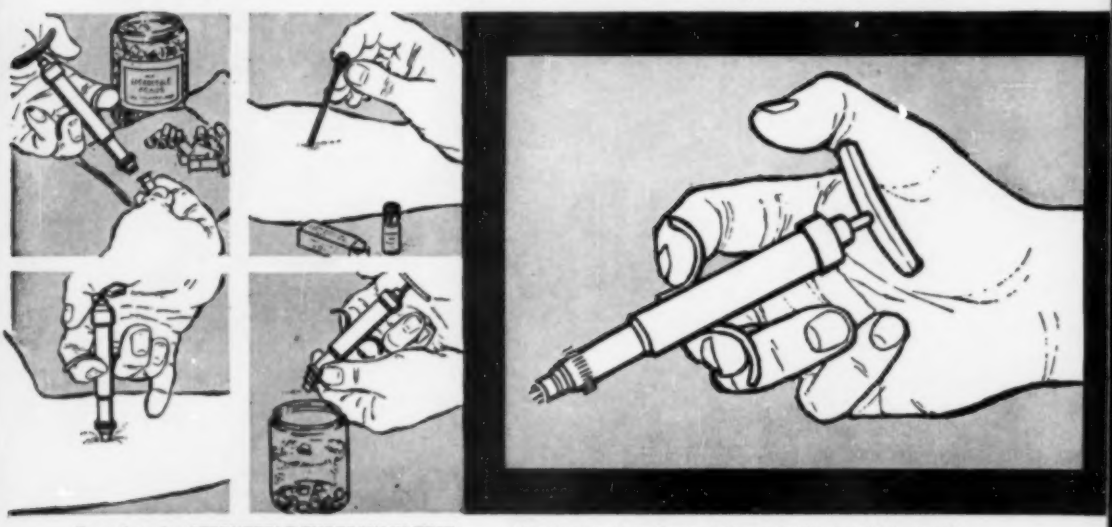

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